MHIIb Gene Diversity and Sexual Selection in the Potbellied Seahorse *(Hippocampus abdominalis)*

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Summary

Models of sexual selection theory have traditionally been based on the assumption of discrete sex roles, with a competitive and a choosy sex. In such models, sexual selection is thought to act chiefly on the competitive sex, due to preferences of the choosy sex for particular traits. An increasing number of studies have shown that the mate preferences of the competitive sex may also influence mating patterns, suggesting that existing models of sexual selection may be incomplete. The major histocompatibility complex (MHC/MH) is one of the best characterized regions of the genome, and has served as an important model of how variable selection pressures can influence patterns of genetic variation. MHC is not only an essential part of the vertebrate adaptive immune system, but also plays an important role during mate choice in many vertebrate species, often leading to disassortative mating on the basis of MHC similarity. The exceptionally high genetic diversity of MHC loci is thought to be maintained by a combination of natural and sexual selection.

While female mate choice in fishes is clearly influenced by MH genes, it remains unclear whether males can also detect and use MH odor cues during mating. In this thesis, I investigated the role of sexual selection in shaping MH diversity in the sex-role reversed potbellied seahorse (*Hippocampus abdominalis*), a species with a highly developed form of male parental care and female competition for mates. I focused on the genes of the MH class II beta-chain (MHIIb), the best characterized group of major histocompatibility genes in bony fishes.

In **chapter 1**, a single MHIIb gene was identified in *H. abdominalis* using targeted gene sequencing and genome walking. cDNA sequencing and a 454 transcriptome screen confirmed the existence of a single expressed locus of MHIIb in this species, and parent-offspring comparisons demonstrated that this locus is inherited according to Mendelian expectations. The expression of this gene in male brood pouch tissue suggests that MH genes may be immunologically active during male pregnancy in the seahorse. Sequences of the complete peptide binding region (PBR) in 101 individuals collected from wild and captive-bred populations displayed an excess of nonsynonymous over synonymous substitutions, a pattern of genetic diversity maintained by a combination of positive selection and intralocus recombination. Cross-

species comparisons showed that intralocus MHIIb PBR diversity of the seahorse is similar to that detected in species carrying a single copy of this locus, and exceeds that of species with multiple MHIIb loci, a pattern which likely reflects the homogenizing effects of interlocus gene conversion in species carrying multiple gene copies. The results of this chapter provide the first evidence indicating that sex-role reversed species such as the potbellied seahorse are capable of maintaining the high levels of MH diversity typical in species with conventional sex roles.

The high genetic diversity of major histocompatibility genes is thought to be influenced by a combination of mutation, gene duplication and loss, recombination, selection and drift. The existence of a single locus in the seahorse simplifies investigations of the impact of these mechanisms, and the evolution of the complete seahorse MHIIb gene was explored in **chapter 2**. Full gene sequences were obtained from ten individuals originating from multiple populations using direct sequencing and cloning. Despite the high levels of variability in the PBR region of the seahorse (see chapter 1), levels of diversity outside this region in both exons and introns are remarkably low, and a complete absence of synonymous substitutions outside the PBR appears to reflect the effects of repeated selective sweeps associated with the PBR of the gene. Surprisingly, individuals carrying MH alleles identical in the PBR often showed variability outside this region, suggesting either the accumulation of neutral variation over time in conserved allelic lineages or convergent evolution. Short sequence tracts were found to be conserved between otherwise divergent sequences, and a significant signature of intralocus recombination in the seahorse MHIIb gene was found both in and outside the PBR, characteristics consistent with a model of intralocus gene conversion. A heavily biased nucleotide composition in both exons and introns might also contribute to the lack of synonymous variation outside the PBR of this gene.

Diversity at MHC genes is thought to be maintained by a combination of parasite-mediated selection and mate choice. In teleosts, MH-based mate choice has been shown in females, but not in males, a phenomenon which could reflect the fact that the majority of species studied have conventional sex roles. The role of sexual selection in shaping MHIIb diversity in the sex-role reversed potbellied seahorse was

studied in **chapter 3**. Focal individuals were first provided with olfactory cues derived from stimuli differing in MHIIb-dissimilarity to the focal seahorses to test the role of MHIIb olfactory cues in mating preferences. A second experiment studied the combined effects of visual and olfactory cues on mating preferences. Finally, reproductive behavior in a large experimental population was assayed using molecular analyses, providing a means to test how MHIIb diversity and sex-specific preferences influence realized mating behavior. The results of these three experiments demonstrated that females preferred and mated with MHIIb-dissimilar stimuli, but showed no evidence of size preference, while males preferred and mated with large-bodied females, but showed no evidence of MH-based preferences. Interestingly, both male and female preferences influenced mating behavior in our experimental population. Male mating success increased significantly with MH-diversity, while female mating success increased significantly with body size. These results suggest the presence of mutual mate choice in *H. abdominalis* and call into question the dichotomous view of sex roles, with one sex being choosy and the other sex being competitive. The absence of male MH-based preferences in a sex-role reversed species supports previous work on species with conventional sex roles and suggests that the use of MH-based olfactory cues in teleosts during mating is restricted to females. While the reasons for this sex-specific difference are as yet unknown, this pattern may reflect sex-specific differences in fitness-related traits associated with reproduction or sex-specific differences in olfactory capacity similar to those detected in mammals.

In conclusion, this thesis demonstrates that sexual selection contributes to the high MHIIb PBR diversity observed in the potbellied seahorse via female preferences for MH dissimilar individuals, whereas the remainder of the gene shows remarkably low diversity. The results of the data presented here demonstrate that care must be taken when behavioral observations of choosiness and competition are used to infer sex roles, as experimental tests under more realistic scenarios, incorporating the preferences of both sexes and multiple mating cues are likely to result in a more nuanced appreciation of how sexual selection acts in natural populations, and how the interaction of natural and sexual selection contributes to patterns of population genetic diversity.

Zusammenfassung

Modelle zur sexuellen Selektion basierten traditionell auf der Annahme getrennter Geschlechterrollen, mit einem konkurrierenden und einem wählerischen Geschlecht. In diesen Modellen wird aufgrund der Preferenzen des wählerischen Geschlechts für bestimmte Merkmale angenommen, dass sexuelle Selektion vor allem auf das konkurrierende Geschlecht wirkt. Eine zunehmende Anzahl an Studien hat jedoch gezeigt, dass auch Partnerpreferenzen des konkurrierenden Geschlechts einen Einfluss auf Paarungsmuster haben können, was darauf hindeutet, dass derzeitige Modelle zur sexuellen Selektion unvollständig sein könnten. Der Haupthistokompatibilitätskomplex (MHC/MH) ist eine der am besten charakterisierten Regionen des Genoms und diente als wichtiges Modell um zu untersuchen wie variable Selektionsdrücke die genetische Vielfalt beeinflussen können. Der MHC ist nicht nur ein bedeutender Teil des erworbenen Immunsystems in Wirbeltieren, sondern er spielt auch eine wichtige Rolle bei der Partnerwahl in vielen Wirbeltierarten und führt oft zu disassortativen Paarungen basierend auf der MHC-Ähnlichkeit. Die ungewöhnlich grosse genetische Diversität von MHC-Genen wird vermutlich durch eine Kombination von natürlicher und sexueller Selektion erhalten.

Während weibliche Partnerwahl in Fischen offensichtlich durch MH-Gene beeinflusst wird, ist unklar, ob Männchen auch in der Lage sind MH-Geruchsspuren während der Paarung zu erkennen und zu nutzen. In der vorliegenden Doktorarbeit habe ich den Einfluss der sexuellen Selektion auf die MH-Diversität im geschlechterrollengetauschten Dickbauchseepferdchen (*Hippocampus abdominalis*) untersucht, eine Art mit einer hochentwickelten Form von väterlicher Fürsorge und weiblichem Konkurrenzkampf um Partner. Ich habe dabei die Gene der MH-Klasse II-Betakette (MHIIb) untersucht, die am besten charakterisierte Gruppe von MH-Genen in Knochenfischen.

Durch gezielte Gensequenzierung und Genome Walking wurde in **Kapitel I** ein einzelnes MHIIb-Gen in *H. abdominalis* identifiziert. Das Sequenzieren von cDNA und eine 454 Transkriptomanalyse bestätigten das Vorhandensein eines einzelnen, exprimierten MHIIb-Gens in dieser Art und Eltern-Kind Vergleiche zeigten, dass dieses Gen nach Mendelschen Erwartungen vererbt wird. Die Expression dieses Gens in

männlichem Bruttaschengewebe deutet darauf hin, dass MH-Gene während der männlichen Schwangerschaft in Seepferdchen immunologisch aktiv sein könnten. Sequenzen der kompletten Proteinbinderegion (PBR) von 101 Individuen, die von natürlichen und gezüchteten Populationen stammen, wiesen einen Überschuss an nicht-synonymen (dN) zu synonymen (dS) Substitutionen auf. Dieses Muster an genetischer Diversität wird durch positive Selektion und intragene Rekombination erzeugt. Vergleiche mit anderen Arten zeigten, dass die intragene MHIIb-PBR-Diversität im Seepferdchen ähnlich der in Arten mit einem einzelnen MHIIb-Gen ist, und dass sie die Diversität in Arten mit mehreren MHIIb-Genen übersteigt. Dieses Ergebnis spiegelt wahrscheinlich die homogenisierende Wirkung von intergener Genkonversion in Arten mit mehreren Genkopien wider. Die Ergebnisse dieses Kapitels liefern erste Beweise, die darauf hindeuten, dass geschlechterrollengetauschte Arten, wie das Seepferdchen, in der Lage sind die hohen Level an MH-Diversität beizubehalten, die typisch für Arten mit normalen Geschlechterrollen sind.

Die grosse genetische Diversität von MHC-Genen wird vermutlich durch eine Kombination von Mutation, Genduplikation und –verlust, Rekombination, Selektion und Drift beeinflusst. Die Existenz eines einzelnen Gens im Seepferdchen vereinfacht Untersuchungen zur Wirkung dieser Mechanismen. In **Kapitel II** wurde die Evolution des kompletten MHIIb-Genes im Seepferdchen untersucht. Komplette Gensequenzen wurden durch direktes Sequenzieren und Klonieren von 10 Tieren, die aus unterschiedlichen Populationen stammen, erhalten. Trotz der hohen Variabilität in der PBR-Region im Seepferdchen (siehe Kapitel I) sind Diversitätslevel ausserhalb dieser Region in Exons und Introns erstaunlich gering und ein komplettes Fehlen von synonymen Substitutionen ausserhalb der PBR scheint die Folgen von wiederholten selektiven Sweeps widerzuspiegeln, die mit der PBR des Gens verbunden sind. Erstaunlicherweise zeigten Individuen mit in der PBR identischen MH-Allelen Variabilität ausserhalb dieser Region, was entweder auf die Anhäufung neutraler Variation in konservierten Allel-Linien über die Zeit hinweg hindeutet, oder auf konvergente Evolution. Sowohl kurze konservierte Sequenzabschnitte zwischen ansonsten unterschiedlichen Sequenzen, als auch signifikante Anzeichen für intragene

Rekombination in und ausserhalb der PBR wurden im Seepferdchen MHIIb-Gen gefunden, Merkmale, die mit einem Model zur intragenen Genkonversion übereinstimmen. Ausserdem könnte eine stark unausgewogene Nukleotid-zusammensetzung in Exons und Introns zusätzlich zum Fehlen von synonymer Variabilität ausserhalb der PBR dieses Gens beitragen.

Die Diversität von MHC-Genen wird vermutlich durch eine Kombination von Selektion durch Parasiten und Partnerwahl erhalten. In Knochenfischen wurde MH-basierte Partnerwahl in Weibchen, aber nicht in Männchen aufgewiesen, ein Phänomen, das die Tatsache widerspiegeln könnte, dass die Mehrzahl an untersuchten Arten konventionelle Geschlechterrollen aufweist. In **Kaptel III** wurde die Beteiligung der sexuellen Selektion an der Bildung von MHIIb-Diversität im geschlechterrollen-getauschten Dickbauchseepferdchen untersucht. Zuerst wurden den betreffenden Individuen Geruchssignale dargeboten, die von Stimuli mit unterschiedlicher MHIIb-Unähnlichkeit zum wählerischen Tier stammten, um die Rolle von MHIIb-Geruchssignalen in Partnerpreferenzen zu testen. In einem zweiten Experiment wurde die kombinierte Wirkung von visuellen und olfaktorischen Signalen auf Partnerpreferenzen untersucht. Anschliessend wurde mithilfe von molekularen Analysen das Reproduktionsverhalten in einer grossen experimentellen Population untersucht, was ein Mittel zur Überprüfung der Einflüsse von MHIIb-Diversität und geschlechtsspezifischen Preferenzen auf das tatsächliche Paarungsverhalten darstellt. Die Ergebnisse dieser drei Experimente zeigten, dass Weibchen MHIIb-unähnliche Stimuli vorzogen und sich mit diesen paarten, aber keine grössenbasierte Preferenz aufwiesen, während Männchen grosswüchsige Weibchen bevorzugten und sich mit diesen paarten, aber keine Hinweise auf MH-basierte Preferenzen zeigten. Sowohl männliche als auch weibliche Preferenzen beeinflussten das Paarungsverhalten in unserer experimentellen Population. Der Paarungserfolg der Männchen stieg signifikant mit ihrer MH-Diversität an, während der Paarungserfolg der Weibchen signifikant mit ihrer Körpergrösse zunahm. Unsere Ergebnisse deuten auf beidseitige Partnerwahl in *H. abdminalis* hin und stellen die dichotome Betrachtungsweise von Geschlechterrollen mit einem wählerischen und einem konkurrierenden Geschlecht in Frage. Das Fehlen

von männlicher MH-basierter Partnerwahl in einer geschlechterrollengetauschten Art bekräftigt frühere Studien an Arten mit normalen Geschlechterrollen und deutet darauf hin, dass der Gebrauch von MH-basierten Geruchsinformationen während der Paarung in Knochenfischen auf die Weibchen beschränkt ist. Während die Gründe für diesen geschlechtsspezifischen Unterschied zur Zeit noch unbekannt sind, könnte dieses Muster geschlechtsspezifische Unterschiede in fitnessrelevanten Merkmalen verbunden mit der Fortpflanzung widerspiegeln, oder geschlechtsspezifische Unterschiede im Geruchssinn ähnlich der in Säugern.

Zusammenfassend zeigt diese Doktorarbeit, dass sexuelle Selektion zur hohen MHIIb-PBR-Diversität im Dickbauchseepferdchen beiträgt, und zwar durch weibliche Preferenz MH-unähnlicher Partner, während der Rest vom Gen eine extrem geringe Diversität zeigt. Die Ergebnisse der hier presentierten Daten zeigen auch, dass Vorsicht geboten ist, wenn Beobachtungen wählerischen und konkurrierenden Verhaltens genutzt werden um auf Geschlechterrollen zu schliessen, da experimentelle Untersuchungen unter realistischeren Bedingungen, die die Preferenzen beider Geschlechter und vielfältige Reproduktionssignale beinhalten, sehr wahrscheinlich zu einer genaueren Einschätzung sowohl der Wirkung sexueller Selektion in natürlichen Populationen, als auch des Beitrags von sexueller und natürlicher Selektion zur populationsgenetischen Diversität führen werden.

GENERAL INTRODUCTION

Sexual selection

Sexual selection is an important mechanism shaping patterns of genetic diversity. It reflects the differential reproductive success among individuals, due to heritable genetic variation in a trait that influences the ability to get mates (Jennions & Petrie 1997; Neff & Pitcher 2005; Lehmann et al. 2007). While direct benefits, such as food or parental care, provide clear criteria for choice (Moller & Jennions 2001), indirect benefits are more difficult to assess. Indirect fitness benefits provide an increased genetic quality of offspring and may result from either mate choice for good genes, where specific homozygous genotypes are preferred, or mate choice for compatible genes, where mate preferences vary between individuals due to an interaction between maternal and paternal genotypes (Irwin & Taylor 2000; Kokko et al. 2003; Neff & Pitcher 2005). Evidence for good genes comes from a variety of systems, including guppies (*Poecilia reticulata*), where paternal body size has been shown to correlate with fecundity of female offspring (Reynolds & Gross 1992). Another example are peacocks (*Pavo cristatus*), where male tail feather eye size correlates with offspring survivorship (Petrie 1994). These studies suggest that condition-dependent phenotypic traits may reflect underlying genetic variation (Rowe & Houle 1996). Support for the compatible genes hypothesis has been found in bluethroats (*Luscinia svecica*), where females engaging in extra-pair copulations produced more immunocompetent offspring with the extra-pair males than with their social mates (Johnsen et al. 2000). One of the best examples for the indirect genetic benefits of mate choice comes from the genes of the major histocompatibility complex (MHC), which provide evidence for both the good genes (Wedekind et al. 2004; Eizaguirre et al. 2009a), and the compatible genes hypotheses (Wedekind et al. 1995; Wedekind & Furi 1997; Penn et al. 2002).

Sexual selection research has heavily emphasized female preferences for male traits, with female traits being considered a by-product of sexual selection on males (Amundsen 2003; Neff & Pitcher 2005; Lehmann et al. 2007). Most work in this area has been performed on species with conventional sex roles, where females are considered to be choosy and males compete for access to mates (Eens & Pinxten 2000). In recent years, evidence has accumulated indicating that males may also show preferences for

female traits during mating (Amundsen & Forsgren 2001; Berglund & Rosenqvist 2001a; Berglund & Rosenqvist 2001b; Gillingham et al. 2009). Sex-role reversed species, in which males are the choosy sex and females compete (Eens & Pinxten 2000), offer a great opportunity to extend tests of sexual selection theories beyond their traditional framework and to test the importance of male mate choice in sexual selection. As a major step in this direction, this thesis explores how sexual selection influences patterns of MHC genetic variation in a sex-role reversed fish species, the potbellied seahorse (*Hippocampus abdominalis*) (Wilson & Martin-Smith 2007).

The major histocompatibility complex

Major histocompatibility complex (MHC) genes encode molecules that are an essential part of the vertebrate adaptive immune system. MHC class I molecules are located on all nucleated cells and are activated following the binding of antigens synthesized within the host (e.g. viruses). These proteins are then presented to cytotoxic CD8-T-cells, which become activated and destroy the infected cell (Janeway et al. 2002). MHC class II molecules, on the other hand, are restricted to specialized antigen-presenting cells (such as B-cells and macrophages). CD4-T-cells are activated by exogenously produced peptides (e.g. bacteria) bound to class II molecules. These T-cells then activate B-cells which produce specific antibodies against the presented antigen. T-cells bound to MHC class II : antigen complexes on macrophages initiate the destruction of antigens in the macrophage vesicles (Janeway et al. 2002).

The investigation of MHC genes in a diversity of vertebrates indicates that these genes have higher levels of diversity than any other gene family (Janeway et al. 2002). Despite consistently high levels of variation, there are major differences in the structure and organization of MHC genes in different vertebrate groups. While MHC genes are physically linked in mammals and Chondrichthyes, class I and II genes are unlinked in the Actinopterygii (Sato et al. 2000). Consequently, MHC gene classes can evolve independently in fishes, resulting in a potentially higher MHC diversity in this group.

Due to the lack of linkage of class I and II genes in Actinopterygians, the use of the term MH for this group has been recommended by Stet et al. (2003).

Selection pressures affecting MHC genes

MHC genes are a textbook-example for the maintenance of polymorphism by balancing selection. MHC polymorphism is thought to be caused by frequency-dependent selection, whereby the optimal allele in each generation is influenced by its relative frequency, an example of the Red Queen hypothesis (Milinski 2006). The advantage of heterozygote MHC genes lies in the increase of the number of different antigens that can be detected by an individual's immune system. Peptide binding sites (PBS) encode a pocket in the MHC molecule that allows the binding of specific antigens, and the peptide binding region (PBR) typically exhibits the highest sequence polymorphism within the gene (Janeway et al. 2002). Purifying selection is thought to act on codons outside the PBR of MHC genes and is indicated by a significantly smaller number of non-synonymous (amino acid changing) to synonymous (silent) substitutions in these regions (Nei & Gojobori 1986).

MHC protein structure models (e.g. by X-ray structure analysis) are currently only available for mammalian MHC genes (Brown et al. 1993). Consequently, the assignment of residues of the PBR in other groups has been done by comparisons with human sequences (homology modeling). In humans, PBS inferred from X-ray structure analysis possess a high amino acid substitution rate (Reche & Reinherz 2003) and indications of positive selection (Hedrick et al. 1991). While the PBR of MHC genes is thought to be evolutionary conserved, individual peptide binding sites may vary among species. In the PBR of classical MH class I genes in the three-spined stickleback (*Gasterosteus aculeatus*), for example, only 13 out of 21 positively selected sites matched to the 34 human PBS identified by X-ray analysis (Schaschl & Wegner 2006), highlighting the fact that homology modeling may not be an adequate method to identify lineage-specific residues of the PBR. Putative PBS in non-model organisms may

be localized by identifying sites with high nonsynonymous substitution rates and signs of positive selection (Schaschl & Wegner 2006).

Studies on three-spined sticklebacks, the bony fish for which MH genes have been best characterized, provide evidence that gene duplication, recombination and inter- and intra-locus gene conversion are important mechanisms driving MH allelic diversity in this species (Reusch et al. 2004; Reusch & Langefors 2005; Schaschl & Wegner 2007). Further studies on *G. aculeatus* indicate that MH diversity is important for both parasite resistance and mate choice (Reusch et al. 2001; Aeschlimann et al. 2003; Milinski 2003; Wegner et al. 2003; Kurtz et al. 2004). In contrast to the heterozygote advantage hypothesis (Milinski 2006), thought to operate in most species, an optimal number of MH variants in the stickleback is correlated with a minimal parasite load. This pattern is thought to reflect the balance between the number of pathogens recognized by different MHC molecules and the loss of self-reactive T-cell clones by negative selection during T-cell maturation (Janeway et al. 2002; Milinski 2006). Consequently, individual mate choice preferences in this species may vary significantly among females, depending on their own MH genotype (Reusch et al. 2001).

Olfaction and mate choice

Inbreeding avoidance, the avoidance of mating with close relatives, may effectively maintain high levels of genetic diversity in vertebrates. Odor cues provide information on kinship based, in part, on MHC allelic composition, allowing the avoidance of potentially closely related individuals carrying a similar MHC haplotype during mate choice (Milinski 2006). Inbreeding avoidance is important, as mating with close relatives causes a reduction in genetic diversity and may reduce offspring fitness (Keller & Waller 2002).

When MHC molecules are released from the cell surface, they are dispersed with body fluids and dissociate their ligands, which can then interact with receptors in sensory neurons (Leinders-Zufall et al. 2004; Milinski et al. 2005). In mammals, these neurons are situated in the vomeronasal organ, a sexually dimorphic organ implicated in

male- and female-specific behaviour in mice (Sagovia & Guillamon 1993; Kimchi et al. 2007).

The best evidence that the genetic constitution of a mate can be detected via olfaction by the choosing individual comes from surveys of mammals (Penn & Potts 1999). Both female and male mice, for example, show MHC-dependent mate preferences (Penn & Potts 1999). Almost all studies have detected an influence of MHC or increasing heterozygosity of MHC genes during mate choice (e.g. Penn & Potts 1999; Milinski 2006), but most studies on olfaction and mate choice have focused on females as the choosy sex, disregarding the potential importance of male mate preferences. Olfactory abilities (e.g. detection, sensitivity and discrimination) in mammals appear to be higher in females than in males (Dorries et al. 1995; Baum & Keverne 2002; reviewed in Good & Kopala 2006; but see Wesson et al. 2006), suggesting the potential for sex-specific differences in the importance of olfactory cues during reproduction.

Fish do not possess a vomeronasal organ, but cells with characteristics similar to mammalian olfactory neurons exist in their olfactory rosette (Hansen et al. 2004). This rosette consists of epithelial lamellae, containing all three morphological types of olfactory neurons (ciliated, microvillous, and crypt cells) (Hansen et al. 2004). Recent studies suggest that male and female teleosts may respond differently to olfactory cues (Lastein et al. 2006; Neff et al. 2008; Ratterman et al. 2009). While it has been shown that mate choice decisions by females depend on olfactory cues signalling MH diversity (Reusch et al. 2001; Forsberg et al. 2007; Neff et al. 2008; Eizaguirre et al. 2009b; Agbali et al. 2010) and on kinship (Olsen et al. 1998; Plenderleith et al. 2005), it is unknown whether male teleosts can also use MH-based olfactory cues during mate choice, something which could influence the pattern of genetic diversity at these loci.

"MHIIb Gene Diversity and Sexual Selection in the Potbellied Seahorse (*Hippocampus abdominalis*)": This thesis

In this thesis, I investigated the role of sexual selection in structuring patterns of genetic diversity of MH class II beta-chain genes (MHIIb) in the potbellied seahorse

(*Hippocampus abdominalis*). As outlined above, MH diversity is thought to be maintained in part by female mate choice (Forsberg et al. 2007; Neff et al. 2008), and sex-specific differences in olfactory abilities might explain the lack of male MH-based mate choice found in fishes. Alternatively, if both sexes are able to detect MH-based olfactory cues, but mating decisions are dictated by the choosy sex, sex-role reversed species, with male choice and female-female competition, might be expected to show levels of MH variation similar to species with conventional sex roles. To test this hypothesis, I have investigated MH genetic diversity in the potbellied seahorse, a non-model teleost species with high paternal investment and sex-role reversal (Wilson & Martin-Smith 2007). Using a combination of genetic data and behavioural experiments, I investigated the molecular diversity and evolution of the seahorse MHIIb gene (chapters I and II) and clarified the role of sexual selection in shaping this diversity (chapter III).

Study species and system

The teleost family Syngnathidae (seahorses and pipefish) is an appropriate model system to study questions related to the role of mate choice in maintaining MH diversity due to its diverse reproductive behaviour. Both conventional and sex-role reversed species exist within the family, and sex-role reversal has evolved several times independently in this group (Wilson et al. 2003). Sex-role reversal results in female-female competition for mates and may lead to the evolution of secondary sexual characters in females (Eens & Pinxten 2000). A second unique characteristic of the Syngnathidae is their high level of paternal investment. Eggs transferred by the female during mating are incubated in brood pouches on the males' abdomen or tail. These brood pouches are diverse in structure, ranging from simple ventral gluing areas to fully enclosed pouches (Wilson et al. 2001). Previous studies on syngnathid fishes have focused on the role of phenotypic and environmental traits (e.g. body size, sex ratio) in sexual selection (Berglund et al. 2005; Mattle & Wilson 2009). While several recent studies have investigated the role of olfactory cues during mating in pipefish species

(Ratterman et al. 2009; Sundin et al. 2010; Lindqvist et al. 2011), MHC genes have not yet been studied in this group.

The potbellied seahorse, *Hippocampus abdominalis*, is a large marine species inhabiting the temperate waters around Tasmania, Southeast Australia and New Zealand. It exhibits a complex form of male parental care with a fully enclosed brood pouch in which embryos are aerated, osmoregulated, protected and nourished until hatching (Stölting & Wilson 2007). The potbellied seahorse forms large breeding aggregations (Martin-Smith & Vincent 2005) and despite social promiscuity, *H. abdominalis* mates monogamously within broods (Woods 2003; Wilson & Martin-Smith 2007). While sexual selection in *H. abdominalis* has been shown to be influenced by female body size (Mattle & Wilson 2009), the importance of olfactory cues during mate choice in the seahorse has not yet been investigated.

Outline of the thesis

In **chapter I** of this thesis, I characterized the MHIIb gene in the potbellied seahorse. MH genes have not yet been identified in syngnathid fishes and their diversity in sex-role reversed species such as the seahorse is unknown. I employed targeted gene sequencing, genome walking and 454 transcriptome screening to identify the complete MHIIb gene sequence and clarify the number of MHIIb loci in the seahorse. cDNA sequencing was used to confirm gene expression. The mode of inheritance of MHIIb alleles in the seahorse was inferred using parent-offspring analysis. The second goal of this chapter was to compare sequence variability in the polymorphic PBR between sex-role reversed and conventionally mating teleost species, in order to test whether MH genetic diversity differs between mating systems.

After investigating the genetic diversity of the functionally active PBR of the seahorse MHIIb gene in chapter I, I broadened my analysis to include the full gene in **chapter II**. Theoretical studies have shown that inter- and intralocus gene conversion are important molecular mechanisms shaping MHC diversity, which are expected to generate contrasting patterns of variability between the PBR and the remainder of the

gene. Unfortunately, previous empirical studies have typically been conducted on multi-locus systems, in which these two factors cannot be easily disentangled. The seahorse, with its single MHIIb gene, offers an ideal system in which to investigate theoretical expectations on the pattern of genetic diversity generated by intralocus gene conversion. Complete gene sequences of teleost MHIIb genes are rare and typically include only a subset of the loci present in a species, complicating comparisons of the molecular evolution of these genes between different vertebrate groups. Complete MHIIb gene sequences were obtained from seahorses of several populations to compare nucleotide diversities between the PBR and the remainder of the gene. I investigated the pattern of nucleotide variability in the seahorse MHIIb gene and explored several mechanisms which may have shaped its evolution.

After clarifying the pattern of genetic diversity of the seahorse MHIIb gene, I was interested in studying the role that sexual selection has played on shaping MHIIb diversity in this species, a question investigated in **chapter III**. In a sequential mate choice experiment, I tested whether male and female seahorses use MHIIb-based olfactory cues during mate choice by presenting focal individuals with odor cues from individuals of different MHIIb-dissimilarity. In a second step, I investigated the relative importance of visual and olfactory cues when presented in concert, revisiting a previous body size-based mate choice study for MH-based preferences, in which olfactory and visual cues were presented together to focal individuals (Mattle & Wilson 2009). Finally, I investigated whether MH- and size-based preferences identified in the first two experiments influence realized mating behavior, establishing a breeding population of *H. abdominalis* and surveying mating behavior via genetic analysis of parentage. In this manner, the relative importance of MHIIb diversity and body size on mate choice and mating success of male and female seahorses could be analyzed. The results of this experiment provide a new perspective on mate choice decisions in this species, and call into question the traditional dichotomous view of sex roles during mating.

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The impact of sex-role reversal on the diversity of the major histocompatibility complex: Insights from the potbellied seahorse (*Hippocampus abdominalis*)

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Abstract

Background

Both natural and sexual selection are thought to influence genetic diversity, but the study of the relative importance of these two factors on ecologically-relevant traits has traditionally focused on species with conventional sex-roles, with male-male competition and female-based mate choice. With its high variability and significance in both immune function and olfactory-mediated mate choice, the major histocompatibility complex (MHC/MH) is an ideal system in which to evaluate the relative contributions of these two selective forces to genetic diversity. Intrasexual competition and mate choice are both reversed in sex-role reversed species, and sex-related differences in the detection and use of MH-odor cues are expected to influence the intensity of sexual selection in such species. The seahorse, *Hippocampus abdominalis*, has an exceptionally highly developed form of male parental care, with female-female competition and male mate choice.

Results

Here, we demonstrate that the sex-role reversed seahorse has a single MH class II beta-chain gene and that the diversity of the seahorse MHIIb locus and its pattern of variation are comparable to those detected in species with conventional sex roles. Despite the presence of only a single gene copy, intralocus MHIIb allelic diversity in this species exceeds that observed in species with multiple copies of this locus. The MHIIb locus of the seahorse exhibits a novel expression domain in the male brood pouch.

Conclusions

The high variation found at the seahorse MHIIb gene indicates that sex-role reversed species are capable of maintaining the high MHC diversity typical in most vertebrates. Whether such species have evolved the capacity to use MH-odor cues during mate choice is presently being investigated using mate choice experiments. If this possibility can be rejected, such systems would offer an exceptional opportunity to study the effects of natural selection in isolation, providing powerful comparative models for understanding the relative importance of selective factors in shaping patterns of genetic variation.

Background

The impact of natural and sexual selection on genetic diversity has been intensively studied in both natural and captive-bred populations (Andersson 1994), but the majority of our current knowledge in this area is derived from species with conventional sex roles, with choosy females and competitive males (Emlen & Oring 1977; Andersson 2005). Sex-role reversed species, in which females compete for mating opportunities and males are choosy (Clutton-Brock & Vincent 1991; Eens & Pinxten 2000), offer exceptional opportunities to investigate central tenets of sexual selection theory and the importance of sexual selection in the maintenance of genetic diversity.

The hypervariable major histocompatibility complex (MHC/MH) has proven to be a powerful model in which to investigate the importance of natural and sexual selection in shaping genetic diversity (Milinski 2006; Piertney & Oliver 2006; Wegner 2008). The MHC is an essential part of the vertebrate adaptive immune system, and includes a suite of more than 200 genes involved in the destruction of infected cells and the antibody response (Janeway et al. 2002). There are two major antigen-presenting groups of MHC molecules, class I and class II genes, which differ in their function, structure and pattern of expression (Janeway et al. 2002). The peptide binding region (PBR) of MHC loci encodes a groove that permits the binding of specific antigens, and typically exhibits the highest sequence polymorphism within the gene (Brown et al. 1993).

The investigation of MHC genes in a diversity of vertebrates indicates that these loci are more diverse than any other gene family (Janeway et al. 2002). Natural selection on MHC is thought to be driven primarily by pathogens, leading to balancing selection that acts on the PBR of MHC genes (Hedrick 1998). Balancing selection operates through either negative frequency-dependent selection, in which the relative fitness of individual alleles is influenced by their frequency (reviewed in Milinski 2006), or via heterozygote advantage. The advantage of MHC heterozygosity lies in the potential increase of the number of different parasite-derived antigens that can be detected by a

MHC-heterozygous individual's immune system (Penn 2002). MHC diversity can be further enhanced by selection on linked genes, due to genetic hitchhiking (Shiina et al. 2006; van Oosterhout 2009). In addition to the importance of MHC genes as an integral part of the adaptive immune system of vertebrates, MHC-mediated odor cues have been shown to be important in mate choice, kin recognition and inbreeding avoidance (Penn & Potts 1999; Reusch et al. 2001; Aeschlimann et al. 2003; Milinski 2003; Forsberg et al. 2007). Disassortative mating is widely believed to promote MHC diversity and to increase the proportion of heterozygote individuals in natural populations (Penn & Potts 1999; van Oosterhout et al. 2006; Spurgin & Richardson 2010). Sexual selection can thus directly contribute to MHC allelic diversity via disassortative mate choice (Penn 2002).

Despite consistently high levels of variation, there are major differences in the genomic organization of MHC genes in different vertebrate groups. While these loci are physically linked in mammals, class I and II genes are unlinked in bony fishes (class Actinopterygii) (Sato et al. 2000; Stet et al. 2003). Due to the lack of linkage of MHC genes in actinopterygians, Stet et al. (2003) have suggested that major histocompatibility genes in these species are most accurately termed MH loci. The unlinked nature of MH genes may provide increased evolutionary flexibility and contribute to enhanced MH diversity in this group. MH gene diversity is highly variable in teleost fishes, and while some species have a single classical MH class II beta-chain gene (MHIIb) (e.g. salmonids: Stet et al. 2002; Harstad et al. 2008), most species have multiple copies of this locus (e.g. sticklebacks: 4-6 copies, Reusch & Langefors 2005; perch: >8 copies, Michel et al. 2009; cichlids: >10 copies, Malaga-Trillo et al. 1998). This variation may be due, at least in part, to ancestral chromosome or genome duplications (Nei & Rooney 2005).

While previous studies on teleosts have shown that both natural and sexual selection structure MH allelic diversity in species with conventional female-based mate choice (Landry et al. 2001; Reusch et al. 2001; Agbali et al. 2010), no study to date has investigated MH variation in sex-role reversed species in which mating decisions are made by the male. Males and females often differ in their ability to detect odor cues (Good & Kopala 2006; Hamdani & Doving 2007), and sex differences in the production,

processing and use of MH-mediated signals are expected to influence the relative efficiency of sexual selection in sex-role reversed and conventionally-mating species, potentially reducing the level of MH variation in species with reversed sex-roles.

The teleost family Syngnathidae (seahorses and pipefish) is a well-suited model system to study questions concerning the relationship between sex roles and MH diversity. Both conventional and sex-role reversed species exist in the family and sex-role reversal has evolved several times independently in this group (Wilson et al. 2003). Studies of wild populations of the potbellied seahorse, *Hippocampus abdominalis,* have found evidence of female-female competition and male mate choice, suggesting that natural populations of this species are sex-role reversed (Wilson & Martin-Smith 2007).

Here, we characterize MH-variation in wild-caught and captive-bred individuals of sex-role reversed populations of the potbellied seahorse, a species with a highly developed form of male parental care. Genome sequencing and transcriptome screening confirm the existence of a single, highly variable copy of the MHIIb locus in this species, with a pattern of variation identical to that detected in species with conventional sex roles. This pattern of genetic variation has been influenced by a combination of intralocus recombination and positive selection on sites believed to be important for peptide binding. MHIIb is expressed in brood pouch tissues of male seahorses, suggesting that these molecules may be functionally active during male pregnancy. Our results indicate that sex-role reversed taxa such as the seahorse are capable of maintaining the high MHC diversity typical of vertebrate species.

Results

The seahorse, *Hippocampus abdominalis*, has a single MHIIb locus

Full-length gDNA sequencing of the seahorse MHIIb locus from a single non-pregnant male identified 2 alleles, closely related to other teleost MHIIb sequences (Blastn: *Hippocampus kuda*: e-value = 0.0, *Hippocampus* sp.: e-value = 2e-100, *Monopterus albus*: e-value = 2e-35, *Archoplites interruptus*: e-value = 1e-33, *Tetraodon nigroviridis*: e-value = 1e-33). The structure of MHIIb in the seahorse is similar to that in other vertebrates, with 6 exons separated by 5 introns of varying length (Figure 1). The total intron length of the 2 full-length alleles differs, resulting in full gene sequences of 3508 bp and 3523 bp, respectively. Intron length variability is concentrated in 3 single-bp repetitive regions (A_n, C_n and T_n) located in introns 2 and 4 (Figure 1).

Complete MHIIb exon 2 sequences were obtained for 96 captive-bred and 5 wild-caught individuals. Irrespective of the primer combination used, a maximum of two alleles were found in all 101 individuals, indicating the existence of a single MHIIb locus in this species. The comparison of parent-offspring MH profiles in 5 families of seahorses confirmed the Mendelian inheritance of the locus in this species (Table 1). A 454-cDNA-library of the potbellied seahorse yielded 36 MHIIb sequences (23 from pregnant pouch tissue (normalized/unnormalized: 18/5), 5 from non-pregnant pouch tissue (2/3), 8 from reference tissues (normalized only)) which could be assembled into a single contig identical to the MHIIb genomic DNA sequence. cDNA sequencing indicated that the MHIIb gene of the seahorse is expressed in muscle, liver and brood pouch tissue.

Sequence polymorphism in the PBR

Sequencing of the highly-variable peptide binding region of the seahorse MHIIb locus identified a total of 17 *H. abdominalis* MHII b1-domain alleles in 101 individuals (Figure 2). 86% of individuals were heterozygous for MHIIb (87 of 101), while 14% were

homozygous, consistent with Hardy-Weinberg expectations (HWE Exact Test: p = 0.08). An analysis of allelic assortment detected 4 different allele combinations more frequently than expected by chance (Figure 3; *Hiab-DAB-E2*03/*03* p = 0.020, **04/*05* p = 0.040, **05/*13* p = 0.029, **07/*08* p = 0.001), but none of these values remained significant after correcting for multiple comparisons (Sequential Bonferroni: p = 0.0003). The 17 alleles include 25 polymorphic nucleotide sites and a total of 17 amino acid differences (Figure 4). Each of the 17 alleles differs by at least one amino acid substitution (Figures 4, 5). All alleles detected in wild individuals (*Hiab-DAB-E2*01, 04, 05, 09, 13, 16* and *17*) were also detected in the captive-bred population. The nucleotide diversity π of the seahorse MHII b1-domain is 0.034. The dataset used for subsequent analyses contains 270 bp of exon 2 (total length: 273 bp), after omitting exon-spanning codons at the 5' and 3' ends of the exon (2 bp and 1 bp, respectively).

A strong signal of positive selection

Only 2 of the 25 nucleotide substitutions detected in exon 2 of the seahorse are synonymous, leading to a dN/dS ratio of 3.7 (dN = 0.041, dS = 0.011, Table 2). A strong signal of positive selection was detected in this region (Z-Test p = 0.02), and 11 of the 17 variable amino acid sites are inferred to be under positive selection ($p < 0.05$, Figure 4) (seahorse sites 4, 6, 8, 17, 43, 60, 63, 67, 70, 74 and 81). A model incorporating positive selection fits the exon 2 dataset significantly better than a neutral model of evolution (M8 vs. M7, LRT = 46.744, df = 2, $p < 0.01$). Non-peptide binding sites in exon 2 show considerably less non-synonymous variation than do PBS (non-PBS dN = 0.018, PBS dN = 0.128) and exhibit no evidence of positive selection (dN/dS = 1.5; Z-Test p = 0.34; Table 2).

Detection of recombination

An allele network based on non-synonymous substitutions was reconstructed to visualize relationships among the 17 unique MHIIb alleles. The network shows no clear spatial structure, consistent with the pattern expected for a single locus (Figure 5a). The

reticulative loop in the network suggests the presence of recombinant variants in the dataset, a hypothesis supported by statistical analyses (RECCO, $p < 0.01$), which indicate that 3 MHIIb alleles are the result of intralocus recombination (*Hiab-DAB-E2*06*: $p = 0.01$, *Hiab-DAB-E2*10*: $p = 0.01$ and *Hiab-DAB-E2*16*: $p = 0.03$). A network without these recombinant alleles is qualitatively similar to the full network, but the placement of *Hiab-DAB-E2*09* shifts in the pruned dataset, reflecting its high level of divergence from the central haplotypes (Figure 5b).

Discussion

The sex-role reversed potbellied seahorse, *H. abdominalis*, has a single MHIIb gene, which exhibits the typical vertebrate pattern of high genetic diversity. The existence of a maximum of 2 MHIIb alleles per individual and the analysis of parent-offspring genotypes in 5 families of seahorses supports the Mendelian segregation of a single locus in this species. The high variability of the b1-domain of this gene, the region interacting with antigens, has been generated and maintained by a combination of positive selection and intralocus recombination, factors which have been shown to influence the pattern of MH variation in species with conventional sex roles (Reusch & Langefors 2005; Wegner 2008). The results of targeted gene sequencing are congruent with a transcriptome screen which indicates that a single copy of this locus is expressed in muscle, liver and brood pouch tissue of the seahorse. The expression of MHIIb in pouch tissue of *H. abdominalis* males suggests that MH molecules may be immunologically active in brood-pouch tissues, and could possibly play a role in immune protection during the development of embryos in the paternal brood pouch (Stölting & Wilson 2007).

Genetic diversity

Previous studies of MHIIb diversity in teleost fishes have demonstrated the exceptionally high diversity of this locus in this group (reviewed in Wegner 2008). These studies have, however, focused on species with conventional sex roles, with female-based mate choice and male-male competition (e.g. *Gasterosteus aculeatus*: Bell & Foster 1994; *Oncorhynchus* spp., *Perca fluviatilis*: Breder & Rosen 1966; *Poecilia reticulata*: Houde 2001). As males and females often differ in their ability to detect olfactory cues (Good & Kopala 2006; Hamdani & Doving 2007), the efficiency of odor-based MHC-mediated choice as a selective mechanism might be expected to differ between sex-role reversed and conventionally-mating species. Disassortative mating is thought to act together with pathogen-mediated selection to maintain MHC diversity (Penn & Potts 1999; Spurgin & Richardson 2010), and species which lack the ability to detect and process MHC-based odor cues are thus expected to exhibit reduced levels of MHC diversity relative to species experiencing both forms of selection. Contrary to this hypothesis, MHIIb diversity in the sex-role reversed seahorse is similar to that detected in other teleosts (see below), suggesting that sex-role reversed species are capable of maintaining the high MH diversity typical in other vertebrates. Both natural and sexual selection are thought to influence MH diversity (Piertney & Oliver 2006), but the observation of high MHIIb diversity in a sex-role reversed species suggests that natural selection may be sufficient to generate this high variability. This hypothesis is currently being investigated using individual-based simulations (Ejsmond MJ, Radwan J and Wilson AB, in prep.). Alternatively, sex-role reversed species may indeed be capable of processing MH-based olfactory cues, something which is currently under investigation in targeted mate choice experiments in the seahorse.

MHIIb gene-copy variation is high in teleosts, and while some teleost fishes have more than 10 functional copies of MHIIb, a small number of species have only a single locus. Perhaps the best studied example of this are the ancestral tetraploid salmonids, who possess a single classical MHIIb gene (Harstad et al. 2008). The high MHII b1-domain diversity of the potbellied seahorse is similar to that found in this group. The seahorse carries a similar number of alleles (*H. abdominalis*: 17 alleles in 101

individuals, *Oncorhynchus gilae gilae*: 5 / 142, *O. tshawytscha*: 12 / 144, *Salmo trutta*: 24 / 180, *O. mykiss*: 88 / 423), but exhibits fewer polymorphic sites (25 variable sites, 6.2% polymorphism) than that found in salmonids (21 – 70 variable sites, 7.7 - 27.2% polymorphism) (Campos et al. 2006; Aguilar & Garza 2007; Neff et al. 2008; Peters & Turner 2008). *H. abdominalis* and salmonids show comparable nucleotide diversities in the PBR-containing b1-domain of exon 2 (*H. abdominalis*: $\pi = 0.034$; *O. gilae gilae*: $\pi = 0.040$, Peters & Turner 2008; *S. trutta*: $\pi = 0.054$, Campos et al. 2006).

As interlocus gene conversion is thought to contribute to the diversity of gene families (Ohta 1998), one might expect to see higher intralocus variability in species carrying multiple MHIIb loci. While species carrying several functional copies of MHIIb possess a higher total number of alleles, intralocus measures of MHIIb PBR diversity in these species are in fact less than those observed in species with only a single locus. Three-spined sticklebacks (*Gasterosteus aculeatus*), an important model system for the study of teleost MH evolution, are thought to carry at least 4 copies of MHIIb (Sato et al. 1998; Reusch et al. 2001; Reusch & Langefors 2005). A recent survey of 48 sticklebacks from locations in Europe and North America detected a total of 31 exon 2 alleles, or ≤ 8 alleles per locus (Reusch & Langefors 2005). Similarly, a survey of Trinidadian guppies, *Poecilia reticulata*, a species with at least 2 MHIIb loci, recovered 18 exon 2 alleles in 56 individuals (alleles per locus ≤ 9) (van Oosterhout et al. 2006). This pattern can also be observed in other species, for example in *Poecilia formosa* (Schaschl et al. 2008) and *Perca fluviatilis* (Michel et al. 2009), with 9 alleles in 29 individuals (≥ 2 MHIIb loci; ≤ 5 alleles per locus) and 28 alleles in 58 individuals (≥ 8 MHIIb loci; ≤ 4 alleles per locus), respectively. Methodological differences in the sample sizes and spatial scales of studies of MH variation complicate comparative analyses of genetic diversity, but the fact that species carrying a single MHIIb locus have levels of allelic variation equal or greater than those detected in species with multiple copies of these loci (see above), suggests that intralocus allelic diversity of the MHIIb PBR does not necessarily increase when more genes are present in a species. It is important to note, that maximal MHC diversity may also be constrained, both by interactions with the autoimmune response (Nowak et al. 1992; Woelfing et al. 2009) and by consistently high levels of interlocus gene

conversion, which may tend to homogenize genetic variation in species carrying multiple copies of these genes (Martinsohn et al. 1999). These factors may, in part, explain the lower than expected levels of MH variation detected in such species relative to species carrying a single copy of these genes.

Peptide binding sites

We detected an excess of non-synonymous substitutions relative to synonymous substitutions in the PBR-encoding b1-domain of the seahorse, a pattern consistent with that found in species with conventional female-based mate choice. Due to the lack of X-ray crystallographic structure analyses of teleost MH genes, PBS in fishes are typically inferred by homology modeling to human MHC loci (Reche & Reinherz 2003). In addition, sites exhibiting a high variability and signatures of positive selection are also putative candidates for peptide binding sites (Hedrick et al. 1991; Piertney & Oliver 2006; Schaschl & Wegner 2006). 17 of the 90 MHII b1-domain sites of the seahorse are variable (19%), and 11 of these variable sites (65%) show evidence of positive selection. 9 of 11 sites correspond to human PBS as inferred by Reche and Reinherz (2003) (Figure 4). While the length of the MHII b1-domain sequenced often differs between studies, several recent studies have analysed site-specific variation in the same 56 amino acid fragment of MHII b1, stretching from position 25 to 80 of the human alignment (Figure 4). A comparison among these studies indicates that the proportion of sites under positive selection in this region is similar between the sex-role reversed seahorse (6/56 = 11%), and conventionally mating salmonids (5-21%, Aguilar & Garza 2007; *Poecilia* spp.: 11-15%, Schaschl et al. 2008; and perch: 22%, Michel et al. 2009), reinforcing the remarkable consistency in the pattern of MH variation among species, despite differences in their sex roles.

Conclusions

We provide the first data on the pattern of MH diversity in the seahorse (*H. abdominalis*), a species with an exceptionally well-developed form of paternal care and male mate choice. The sex-role reversed *H. abdominalis* exhibits levels of MHIIb diversity similar to that detected in species with conventional sex roles. This species has a single functional MH class II beta-chain gene that is expressed in the male brood pouch, suggesting that this gene may be immunologically active in these tissues. The pattern of MHIIb genetic diversity in the seahorse has been influenced by positive selection and recombination, and intralocus genetic diversity in this species exceeds that present in species carrying multiple copies of this gene. Mating experiments are currently being used to determine whether MH-odor cues are used in mate choice decisions in *H. abdominalis*, data which should help to shed light on the relative roles of natural and sexual selection in generating the high levels of MHIIb diversity found in the seahorse.

Methods

Full-length MHIIb gene sequencing

Whole genomic DNA was extracted from muscle tissue of a single *H. abdominalis* individual using a standard proteinase K / phenol-chloroform protocol (Bruford et al. 1998). To characterize MHIIb genes in the seahorse, we first designed primers in conserved regions of the gene. These regions were identified using an alignment of published sequences for 11 teleost species (*Danio rerio* - Dare [GenBank:AAA50043], *Salmo salar* - Sasa [GenBank:AJ439067], *Cyprinus carpio* - Cyca [GenBank:CAD89312, CAA64709], *Tetraodon nigroviridis* - Teni [GenBank:CAF94187], *Oryzias latipes* - Orla [GenBank:BAA94279, BAA94280], *Poecilia reticulata* - Pore [GenBank:Z54077],

Stizostedion vitreum - Stvi [GenBank:AY158837], *Paralichthys olivaceus* - Paol [GenBank:AB126922, AB126923, AY848955], *Gasterosteus aculeatus* - Gaac [GenBank:AY713945], *Hippocampus kuda* – Hiku [GenBank:AY211533], *Takifugu rubripes* – Taru [Ensembl:ENSTRUP00000004737], *Oryzias latipes* – Orla [Ensembl:ENSORLG00000000025]). Sequences were aligned in BioEdit v.7.0.9.1 (Hall 1999) and primers were designed using Primer3 v.0.4.0 (Rozen & Skaletsky 2000). Primers used for MHIIb sequencing are provided in Table 3 and their locations on the seahorse MHIIb gene are indicated in Figure 1.

To amplify MHIIb, we used long-range PCR under the following conditions: 1x ThermoPol reaction buffer (NEB), 1.2 µM dNTPs, 0.9 µM of each primer, 1.5 U of a 1:20 Pfu DNA polymerase (Promega) and Taq DNA Polymerase (NEB) mixture and approx. 60 ng DNA per 30 µL reaction. PCR running conditions involved an initial denaturation at 92°C for 5 min, followed by 35 cycles of 92°C for 30 sec, 58°C for 30 sec and 68°C for 0.5 - 4 min (depending on product length), with a final extension at 68°C for 5 - 15 min.

As the initial primer set provided only a fragment of the MHIIb locus, genome walking was used to complete the sequence using a protocol modified from the Universal GenomeWalker Kit (Clontech). One µg of high-quality genomic DNA was digested separately with 10 U of the enzymes EcoRV (NEB), PvuII (NEB), StuI (NEB), DraI (NEB), AluI (Promega), HincII (NEB) and Cac8I (NEB) according to the manufacturer's recommendations. Purification of digested DNA and adaptor ligation followed the Clontech protocol. Genome walking was performed using a nested PCR approach with 1x ThermoPol reaction buffer, 1 µM dNTPs, 0.4 µM AP1 primer, 0.4 µM gene-specific primer 1, 1 U Taq DNA polymerase (NEB) and 1 µL of the DNA-adaptor-library in a 20 µL reaction volume for the first round PCR. The nested PCR was performed using the same protocol, but with the AP2 primer and a nested gene-specific primer along with 1 µL of a 1:50 dilution of the initial PCR product. Cycling conditions were identical in both PCRs, with 2 min at 92°C, 30 cycles of 30 sec at 92°C, 30 sec at 57°/60°/63°C and 3 min at 68°C.

PCR products were purified for sequencing using either a MultiScreen PCR filter plate (Millipore), gel-purification with the Wizard SV Gel and PCR Clean-Up System (Promega), or via cloning with a Topo TA Cloning Kit (Invitrogen) following the manufacturers' recommendations. 10-20 positive colonies per plate were picked into 25 µL of ddH20, directly PCR-amplified and sequenced. Cloned products were compared to direct sequences generated with several different primer combinations, in order to identify allelic phase and to identify any cloning-mediated PCR artifacts. Purified PCR products were prepared for sequencing by adding 1 µL Big Dye v3.1 Terminator Cycle Sequencing mixture (Applied Biosystems) and 1 µL primer to 2-8 µl of purified product in a 10 µL volume. Cycling conditions were 30 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Ethanol-purified products were sequenced on an ABI 3730 automated sequencer (Applied Biosystems).

Analysis of gene expression and MHIIb copy number

To determine whether MHIIb sequences obtained from genomic DNA represent functional alleles, we amplified and sequenced a partial MHIIb cDNA sequence (exon 2 - 5) from liver, muscle and pouch tissue of a reproductively mature non-pregnant male seahorse. RNA was extracted using TRIZOL® Reagent (Invitrogen) according to the manufacturers' recommendations. One µg of purified RNA was digested with 9 µL of DNase I (Promega) and reverse-transcribed into cDNA with 1 µL ImProm II Reverse Transcriptase (Promega) using 2 µL of a 500 µg/µL solution of a dT-adaptor primer (TAGGAATTCTCGAGCGGCCGCTTTTTTTTTTTT) in 25 µL volume. The program for the RT-PCR followed the manufacturer's recommendations (Promega). 3 µL of a 1:2 dilution of Millipore-purified cDNA was used as template in a PCR reaction with MHIIb-E1F2 and MHIIb-E6R under the standard PCR conditions outlined above.

Genomic DNA and cDNA sequencing indicate that *H. abdominalis* possesses a single functional MHIIb gene (see below). To further explore this pattern, we screened cDNA libraries of seahorse pouch and reference tissues from pregnant and non-pregnant individuals for the presence of MH genes using 454 sequencing. Briefly, both

normalized and unnormalized cDNA libraries prepared from purified total RNA derived from the pouch tissues of a single pregnant and non-pregnant seahorse, together with a pool of reference tissues from the pregnant individual (brain, gills, liver, heart, kidney and testes), were individually MID-tagged with a unique sequence identifier. MID-tagged libraries were sequenced using GS FLX Titanium Chemistry (Roche), following the manufacturer's recommendations. A full plate of 454 sequencing yielded a total of 850K filtered reads (average read length 230 bp), 92% of which could be assembled into 38K contigs. The full results of this transcriptome screen will be described in detail elsewhere (Gauthier MEA, Stölting KN and Wilson AB, in prep.).

Characterization of the MHIIb peptide binding region (PBR)

In order to investigate the hypervariable PBR of MHIIb, complete exon 2 sequences were amplified in an additional 100 individuals as part of a larger study investigating MH-based mate choice preferences in the seahorse. Seahorses are listed under Appendix II of the United Nations Convention on the International Trade in Endangered Species (CITES), and the majority of the samples included here originate from a captive-bred population derived from individuals collected from several sex-role reversed Tasmanian populations. The seahorses in this captive-bred population are held in large communal breeding tanks (2,100 L) with 50 males and 50 females per tank, allowing free mate choice (Hawkins R, pers. comm.). This population is genetically diverse (20 - 29 alleles per microsatellite locus; n = 4 loci) and an individual-based assignment test indicates the existence of a single Tasmanian population of captive-bred and wild-caught individuals (Structure: Pr(K=1) = 1; see Additional file 1). A global test of microsatellite data failed to reject the null-hypothesis of Hardy-Weinberg equilibrium in this population (HWE Exact Test: p = 0.21). In addition to 95 individuals from the captive-bred population, we obtained complete exon 2 sequences from 5 wild-caught seahorses from Sydney, Australia (2 individuals collected in 2003) and Tasmania (3 individuals collected from 3 populations in 2003 and 2004). Genomic DNA from these individuals was extracted from fin clips using a DNeasy 96 Tissue Kit (QIAGEN). PCR

products for exon 2 were generated using either primer MHIIb-E1F2 or MHIIb-I1F together with primer MHIIb-I2R4 (see PCR conditions above) and directly sequenced. Sequencing results were identical using either primer combination (data not shown). All private haplotypes were sequenced in a minimum of 2 independent runs in order to minimize the incorporation of PCR artifacts. Degenerate positions in heterozygote sequences were scored using IUPAC nomenclature to facilitate the inference of allelic phase (see below).

MHIIb inheritance

We obtained exon 2 sequences from 47 F1 individuals from 5 families (n = 8-13 per family), to investigate whether MH alleles segregate in a Mendelian fashion. This approach not only demonstrates the mode of inheritance of these loci, but also provides a means to evaluate the reliability of sequence profiles generated for this fragment of the MHIIb gene, through parent-offspring comparisons.

Processing of sequences

Sequence data were assembled using Sequencing Analysis 5.2 (Applied Biosystems). Sequences were aligned with Muscle v.4.0 (Edgar 2004) and verified by eye in BioEdit v.7.0.9 (Hall 1999). To investigate the peptide binding region (PBR), we analysed 270 bp sequences of exon 2 (total length: 273 bp) after omitting the first 2 nucleotides and the final nucleotide of exon 2, to obtain a complete reading frame. As all exon 2 alleles are derived from a single MHIIb locus (see below), they are named *Hiab-DAB-E2*01-17*, following standard terminology (Ellis et al. 2006). MH haplotypes of each individual were inferred from degenerate sequence data using a Bayesian statistical method implemented in PHASE v.2.1 using the default parameters (Stephens & Donnelly 2003), a powerful approach which allows the determination of allelic phase from degenerate electrophoretic profiles (Harrigan et al. 2008). SeqPHASE was used to convert between the PHASE input/output file and the sequence alignment (Flot 2010).

Analyses of sequence polymorphism

DnaSP v.4.90.1 (Librado & Rozas 2009) was used to calculate standard estimates of genetic diversity. To visualize relationships among the different exon 2 alleles and the non-synonymous substitutions separating them, a haplotype network was prepared using TCS v.1.21 (Clement et al. 2000). The conversion of the sequence alignment file into a TCS-file was done with FaBox v.1.35 (Villesen 2007) and the final network was prepared using yED v.3.2.0.1 (yWorks 2009). Tests for Hardy Weinberg equilibrium were performed in Genepop on the web (Raymond & Rousset 1995; Morgan 2000) using the default settings for the Markov Chain search. The analysis of non-random associations of alleles was performed using non-parametric simulations (10,000 permutations), incorporating empirical allele frequencies with the Monte Carlo simulation function in PopTools v.3.0.6 (Hood 2008). 95% confidence intervals of simulated data provided an estimate of expected frequencies of allelic combinations.

Positive selection

dN and dS were calculated using Mega v.4.0.2 (Tamura et al. 2007) under a Jukes-Cantor model. Mega v.4.0.2 (Tamura et al. 2007) was also used to test for positive selection in the dataset, applying a Z-test under a Jukes-Cantor model (10,000 permutations). Site-specific positive selection was inferred using Codeml, implemented in the PAML v.4.2b package (Yang 2007). Codeml tests the goodness of fit of codon substitution models to a dataset using maximum likelihood. Neighbor-joining trees were generated for the 17 exon 2 alleles using Neighbor v.3.5c (Felsenstein 1993) under default settings, as implemented in BioEdit v.7.0.9. We compared the fit of a neutral evolution model with recombination (M7) with one allowing for positive selection (M8), using a likelihood-ratio test (LRT). Most previous studies on patterns of variation at vertebrate MHC loci have used the original human crystallographic structure of MHCIIb prepared by Brown et al. (1993) to infer putative peptide binding sites. More recently, Reche and Reinherz (2003) presented an updated model of human PBS based on a larger sampling of potential peptides. In order to facilitate comparisons with previous

studies, codons of the seahorse PBR were inferred through homology modeling to both of these datasets (see Figure 4), but given the more comprehensive dataset included in the Reche and Reinherz paper (2003), PBS inferences in future studies should place greater emphasis on this work.

Recombination

Recombination in the seahorse exon 2 dataset was tested using the default settings of RECCO v.0.93 (10,000 permutations) (Maydt & Lengauer 2006). The identification of recombinant alleles with RECCO is based on a minimal cost solution, in which the relative cost of obtaining a sequence in an alignment from the other sequences by mutation and recombination is evaluated.

Additional file 1

Figure S1: Genetic structure plot.

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Table 1: Mendelian inheritance of MHIIb in the seahorse.

	Family A				Family B				Family C				Family D				Family E			
Individual	**A1**	**A2**	**A3**	**A4**	**A1**	**A2**	**A3**	**A4**	**A1**	**A2**	**A3**	**A4**	**A1**	**A2**	**A3**	**A4**	**A1**	**A2**	**A3**	**A4**
Father	3	4			2	6			5	13			4	9			2	6		
Mother			4	15			1	8			2	8			6	16			1	11
Juvenile 1		4	4			6	1			13	2		4		6			6		11
Juvenile 2	3			15		6		8	5		2		4			16	2			11
Juvenile 3		4		15		6	1		5		2			9		16		6	1	
Juvenile 4		4		15		6	1			13		8	4		6		2			11
Juvenile 5	3			15		6		8	5			8		9		16		6	1	
Juvenile 6	3			15	2		1			13		8		9	6		2		1	
Juvenile 7	3		4			6		8		13		8		9	6		2		1	
Juvenile 8		4	4			6		8		13		8	4			16	2			11
Juvenile 9	3			15	2		1		5			8								
Juvenile 10	3			15																
Juvenile 11	3		4																	
Juvenile 12	3			15																
Juvenile 13		4		15																

Combinations of MHIIb alleles (A1-4) in parents and F1 offspring of 5 families of seahorses (A-E).

Table 2: Synonymous and non-synonymous substitution rates for exon 2 alleles of the seahorse MHIIb gene.

Locus	Length (bp)	Samples	Alleles	dN	dS	dN / dS
Exon 2	270	101	17	0.041	0.011	3.73*
Exon 2, PBS	72	101	15	0.128	0.009	14.22**
Exon 2, non-PBS	198	101	9	0.018	0.012	1.50^{ns}

Probabilities (*<0.05, **<0.001, ns = not significant) are derived from a Z-test (H1 = positive selection). Peptide binding sites (PBS) refer to the human sites, identified by crystallographic analysis in Brown et al. (Brown et al. 1993).

Table 3: Primers used to amplify and sequence MHIIb in *H. abdominalis.*

Name	Sequence 5'-3'	Location
MHIIb-E1F2	GCCTCCTTTTCCTCACCTTC	Exon 1
MHIIb-I1F	TTGCGACTACACATTCAGCA	Intron 1
MHIIb-I2F2	TTTTTTTATCCCTTAACACTTAGAATACAG	Intron 2
MHIIb-I2F3	CGGGTCAACGAGTTCTCAAC	Intron 2
MHIIb-I2R	ACCAATGATTGTTCGGGTGT	Intron 2
MHIIb-I2R2	TCGGGTGTGATAATGGTCTG	Intron 2
MHIIb-I2R4	GGCGGCTGATTATCATGTTT	Intron 2
MHIIb-I2R5	TTGCGCCAAGGACCGGTTTAATG	Intron 2
MHIIb-E3F	GACGGCGACTGGTACTATCA	Exon 3
MHIIb-E3R	TGATAGTACCAGTCGCCGTC	Exon 3
MHIIb-E3R2	TCTGCTTGGGGTAGAAGTCG	Exon 3
MHIIb-E4R	AAGGCTGGCGTGTTCCAC	Exon 4
MHIIb-I4F	CGGGGGTCTTAAATCCTGTT	Intron 4
MHIIb-E5F	CTTTCCCTGGGAGGCTTC	Exon 5
MHIIb-E6R	TGGGAACCAGAATGCGACC	Exon 6

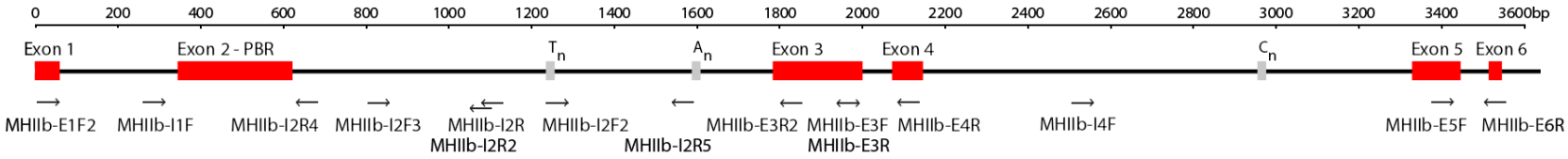


Figure 1: Seahorse MHIIb gene map. The locations of exons, repetitive regions (A_n, C_n and T_n) and primers used for genome walking and sequencing (see table 3) are indicated. The peptide binding region (PBR) of MHIIb is located in exon 2. The full gene sequence has been submitted to GenBank (Accession #: HQ902181 and HQ902182).

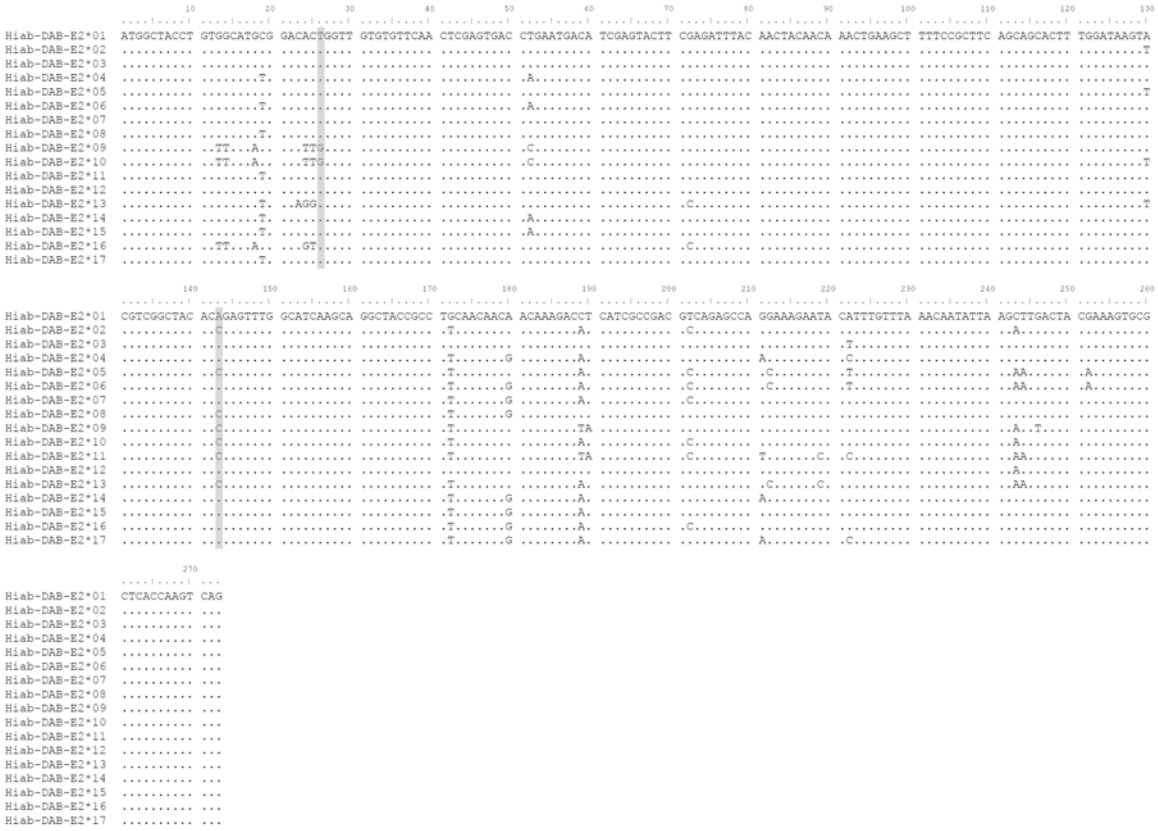


Figure 2 - Nucleotide alignment of exon 2. 17 MHIIb exon 2 sequences were obtained in 101 *H. abdominalis* specimens. Dots indicate identity to the first sequence. Synonymous substitutions are shaded in grey. Exon 2 sequences have been submitted to GenBank (Accession #: HQ902164 – HQ902180).

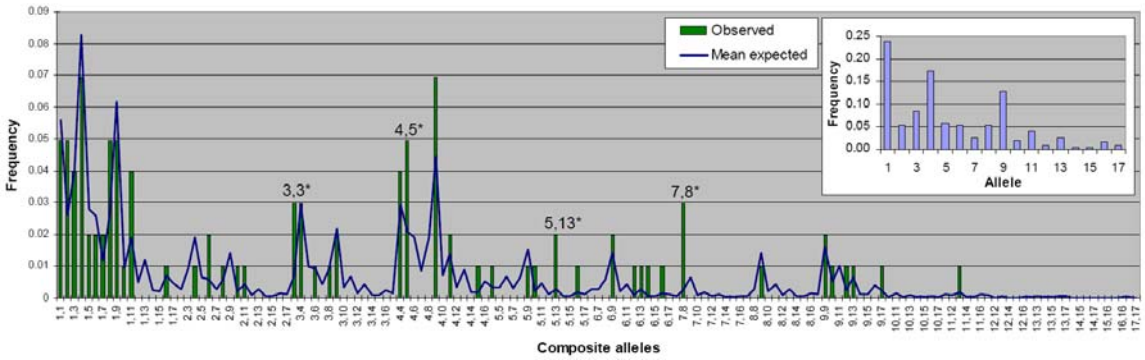


Figure 3 - Observed and expected allelic combinations. Bars represent allelic associations observed in 101 *H. abdominalis* individuals. The mean expected frequencies of allele combinations have been simulated based on the observed allele frequencies (see figure inset). Stars indicate significant deviations ($p < 0.05$) from the mean expectation (N = 10,000 permutations). None of these outliers remain significant after controlling for multiple comparisons.

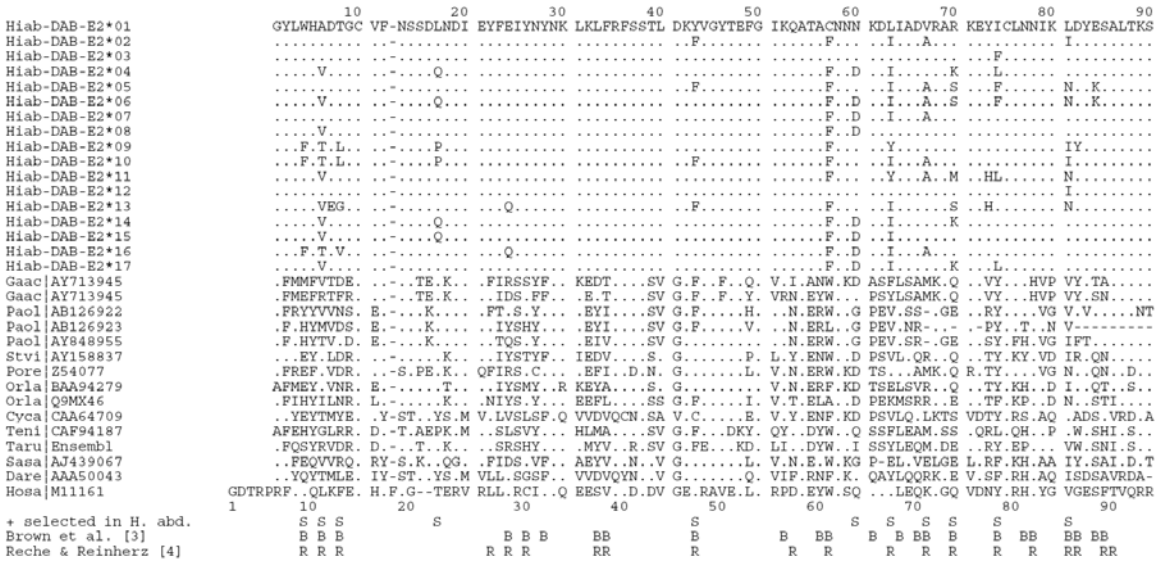


Figure 4 - Amino acid alignment of b1-domain. MHII b1 sequences for *H. abdominalis* (Hiab), *Homo sapiens* (Hosa) and published teleost species (see methods). "S" represents positively selected sites in the seahorse as inferred from the exon 2 dataset, "B" indicates human PBS according to Brown et al. (1993) and "R" reflects human PBS according to Reche and Reinherz (2003). Amino acid positions of the human MHII b1-domain are indicated below the human sequence.

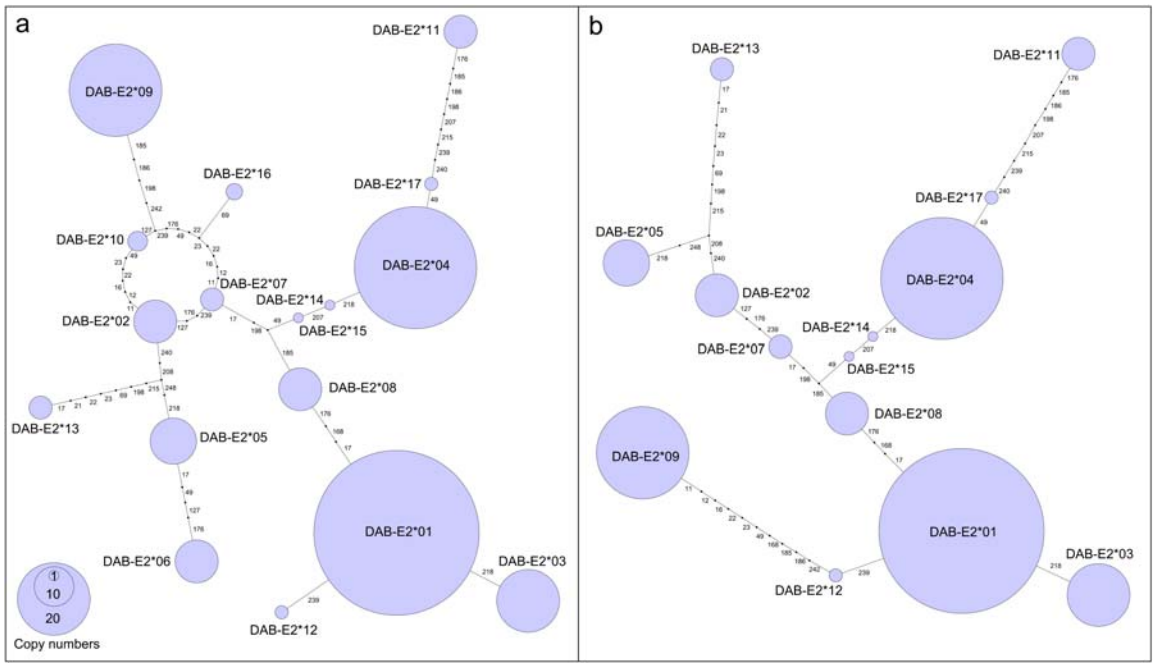
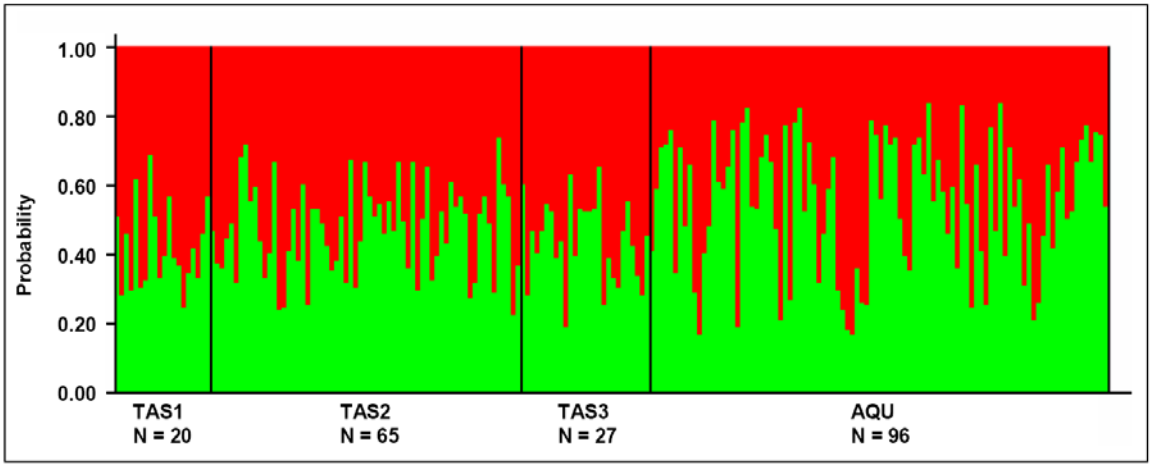


Figure 5 - Allele network of exon 2. MHIIb exon 2 nucleotide sequences for 101 *H. abdominalis* individuals. Circle sizes reflect allele frequencies. The positions of individual non-synonymous substitutions separating sequences are indicated. Figure 5a: All 17 alleles. Figure 5b: Recombinant alleles (RECCO: $p<0.05$) have been removed (*DAB-E2*06*, *DAB-E2*10* and *DAB-E2*16*).



Additional file 1, Figure S1 – Genetic structure plot.

An individual-based analysis of genetic structure based on 4 neutral microsatellites best supports the existence of a single panmictic population (Pr[K=1] = 1.00) of aquaculture individuals (AQU) and samples collected from 3 Tasmanian localities (TAS1-3). The figure shows a structure plot for a two-population model (K = 2) with probabilities of individual assignment to the 2 hypothetical populations.

The evolution of MHC diversity: Evidence of intralocus gene conversion and recombination in a single-locus system

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Submitted to Gene

Abstract

Gene conversion, the unidirectional exchange of genetic material between homologous sequences, is thought to strongly influence patterns of genetic diversity. The high diversity of major histocompatibility complex (MHC) genes in many species is thought to reflect a long history of gene conversion events within and among loci. Theoretical work suggests that intra- and interlocus gene conversion leave characteristic signatures of nucleotide diversity, but empirical studies of MHC variation have rarely been able to analyze the effects of conversion events in isolation, due to the presence of multiple gene copies in most species. The potbellied seahorse (*Hippocampus abdominalis*), a species with a single copy of the MH class II beta-chain gene, provides an ideal system in which to explore predictions on the effects of intralocus gene conversion on patterns of genetic diversity. The genetic diversity of the peptide binding region (PBR) of the gene is high in the seahorse, comparable to other vertebrate species. In contrast, the remainder of the gene shows a total absence of synonymous variation and low levels of intronic sequence diversity, concentrated in 3 short repetitive regions and 1-12 SNPs per intron. The distribution of substitutions across the gene results in a patchwork pattern of shared polymorphism between otherwise divergent sequences. The pattern of nucleotide diversity observed in the seahorse MHIIb gene is congruent with theoretical expectations for intralocus gene conversion, indicating that this evolutionary mechanism has played an important role in MHC gene evolution, contributing to both the high diversity in the PBR and the low diversity outside this region. Neutral variation at this locus may be further reduced due to a biased nucleotide composition and functional constraints.

Introduction

The major histocompatibility complex (MHC / MH in Actinopterygii), a key component of the adaptive immune system, is one of the most diverse gene families in the vertebrate genome (Janeway et al. 2002). The diversity of this gene complex is thought to be created and maintained by a combination of mutation, gene duplication and loss, recombination, selection and drift (Ohta 1991; Yeager & Hughes 1999). The MHC peptide binding region (PBR) encodes a pocket in the molecule that allows the binding of specific pathogen-derived antigens, and this region typically exhibits the highest sequence polymorphism within the gene (Janeway et al. 2002).

Different regions of MHC genes are thought to be influenced by contrasting types of selection. The high diversity of the PBR, for example, is maintained by strong balancing selection (Ohta 1991) and variation at nearby neutral sites may also be maintained by genetic hitchhiking (Hughes 2000). Sexual selection is also thought to contribute to PBR diversity through disassortative mating, where PBR-dissimilar individuals are preferred during mate choice (Penn & Potts 1999). In contrast, the region outside the PBR, critical for the secondary structure of the protein, is subject to purifying selection, with low levels of nonsynonymous substitutions (Hughes & Nei 1989).

Mutations create the diversity on which selection can act, but polymorphism can be further increased by recombination, bidirectional sequence exchange in which both alleles can be altered. Gene conversion, the unidirectional exchange of genetic material between homologous sequences of single or multiple loci, is thought to play an important role in the molecular evolution of gene families such as MHC (Ohta 1982; Hughes 2000; Chen et al. 2007; but see Martinsohn et al. 1999). Gene conversion events are thought to be responsible for both the high diversity of many gene families (e.g. AB0 blood group locus, HLA class II region) (reviewed in Chen et al. 2007) and the patterns of concerted evolution found in many multigene families and highly repeated DNA

sequences (e.g. histone genes, immunoglobulin genes, ribosomal RNA genes) (Rada et al. 1990; Reusch & Langefors 2005; reviewed in Nei & Rooney 2005).

Both inter- and intralocus gene conversion are expected to increase MHC allele number (Ohta 1997; 1999). In the presence of positive selection, even over short evolutionary time scales, neutral variation located away from positively selected sites may be lost, resulting in dN > dS > *d* at loci experiencing gene conversion (Ohta 1997; 1998; 1999). Both types of gene conversion may also lead to a patchwork pattern of nucleotide polymorphism, clusters of shared mutations between otherwise divergent sequences (Parham et al. 1995; Martinsohn et al. 1999; Ohta 2000).

Interlocus gene conversion is expected to lead to increases in diversity (number of heterozygous sites) and the frequency of synonymous (dS), non-synonymous (dN) and intronic (*d*) variation, as new mutants are introduced from divergent loci. Interlocus conversion consequently leads to low dN:dS ratios in the absence of positive selection (Ohta 1998; 1999), and may ultimately lead to the concerted evolution of homologous loci (Ohta 1982). Gene homogenization may occur if variable regions of the gene are repeated exchanged in conversion events (Martinsohn et al. 1999), or if the converted sequences are under directional selection (Hughes 2000).

In contrast to interlocus events, intralocus gene conversion is expected to reduce allelic diversity, as it reshuffles existing variation within the locus (Ohta 1999). Intralocus conversion is also expected to lead to higher dN:dS and dS:*d* ratios in the absence of positive selection (Ohta 1999).

The effect of gene conversion on MHC polymorphism is still under debate (Martinsohn et al. 1999; Nei & Rooney 2005) and several alternative explanations for the nucleotide pattern observed in MHC genes have been suggested. The high MHC allele number and allelic diversity of these genes may be maintained by positive selection alone (Ohta 1991), or by maternal-fetal interactions, the selective disadvantage of allele sharing between mother and offspring (Hedrick & Black 1997; Ohta 1998). Other studies have supported a birth-and-death model of MHC evolution, involving a combination of gene duplication and loss and overdominant selection on the

PBR of the gene (e.g. Hughes & Nei 1989). Nevertheless, simulation studies have shown that overdominant selection alone is insufficient to create a dN:dS ratio larger than one (Ohta 1991; 1997), which requires positive selection at least in the short term (Ohta 1998). Shared polymorphism may result from co-ancestry or convergence (Klein & Figueroa 1986; Kriener et al. 2000), but these mechanisms are unlikely to explain a patchwork pattern of genetic variation in the absence of mutation rates higher than those observed at MHC genes or unrealistically long time periods (>20 Myr ago for a shared motif of 3 amino acids) (Yeager & Hughes 1999; Reusch & Langefors 2005).

Studies on threespined sticklebacks, the bony fish for which MH genes have been best characterized, provide evidence that gene duplication and gene conversion have been important mechanisms driving MH allelic diversity in species with multiple copies of these genes (Reusch et al. 2004; Reusch & Langefors 2005; Schaschl & Wegner 2007). The effects of inter- and intralocus gene conversion and recombination can, however, not be differentiated in multi-locus systems, unless MH alleles can be unambiguously assigned to specific loci (e.g. Reusch et al. 2004), a task which has proven difficult in the MH system (e.g. Sato et al. 1998; Reusch & Langefors 2005; Michel et al. 2009).

Surprisingly, despite the intense analysis of MHCIIb genes in vertebrates (Piertney & Oliver 2006; reviewed in Wegner 2008), full-length gDNA sequences are extremely rare, particularly in fishes. Studies investigating intronic MHIIb diversity have either included full-length sequences of only a subset of loci present in the investigated species (Sultmann et al. 1994; Reusch et al. 2004), or have been restricted to the introns neighboring the PBR (Reusch & Langefors 2005; Michel et al. 2009). Complete MHIIb sequences covering the full genetic diversity present in a species would provide an important tool to compare the molecular evolution of these genes between different groups of vertebrates.

We have studied the evolution of MH genes in the potbellied seahorse, *Hippocampus abdominalis*, a species with a single MH class II beta-chain (MHIIb) gene inherited according to Mendelian expectations (Bahr & Wilson 2011). Complete gene

sequences obtained from multiple individuals show a high ratio of nonsynonymous to synonymous substitutions in the PBR, similar to that found in species with multiple copies of this locus. Contrary to expectations, despite high PBR variation, a complete absence of synonymous variation is found outside the PBR. Sequence diversity in introns is lower than that detected in the PBR, and a patchwork pattern of nucleotide polymorphism was found. The pattern of MHIIb diversity observed in the potbellied seahorse is consistent with theoretical expectations from models of intralocus gene conversion, acting in concert with PBR-positive selection and functional constraints of synonymous sites outside this region.

Materials and Methods

Samples

Complete MHIIb gene sequences were obtained from 10 *H. abdominalis* specimens in order to characterize the distribution of sequence polymorphism within the gene. Two samples were collected from Sydney Harbor (2003) and 3 individuals were collected from 3 Tasmanian locations in 2003 and 2004 (Wilson & Martin-Smith 2007). Mainland Australian and Tasmanian seahorses are genetically distinct (Wilson AB, unpublished data). The remaining 5 samples originate from several generations of an aquaculture population, which was originally derived from seahorses collected from several Tasmanian sites (for details on this population see Bahr & Wilson 2011). Previous analyses of a large sample of this population (N = 101 individuals) revealed a high diversity in the MHIIb PBR, comparable to that observed in other vertebrate species (Bahr & Wilson 2011). The high variability at neutral microsatellite loci observed in *H. abdominalis* (Wilson & Martin-Smith 2007; Bahr & Wilson 2011) suggests that recent population bottlenecks or founder effects are unlikely to have influenced gene diversities in this species (Kim et al. 1999).

Full-length MHIIb gene sequencing

Genomic DNA was extracted from muscle tissue using a standard proteinase K / phenol-chloroform protocol (Bruford et al. 1998). To characterize the full-length MHIIb gene of the seahorse, primers were designed based on the complete MHIIb sequence of a single individual (Hiab-DAB*01/02, Bahr & Wilson 2011). Sequences were aligned using BioEdit v.7.0.9.1 (Hall 1999) and primers were designed using Primer3 v.0.4.0 (Rozen & Skaletsky 2000). Primers used additionally to those previously published (Bahr & Wilson 2011) are provided in Table 1.

To obtain full-length MHIIb sequences, primers MHIIb-E1F2 and MHIIb-E6R were used under the long-range PCR conditions described in Bahr and Wilson (2011). These primers amplify the region between exons 1 to 6 of the gene. 5' and 3' ends of sequences were completed using the primer combinations MHIIb-UTR5F with MHIIb-I2R4 and MHIIb-E5F with MHIIb-UTR3R under the same PCR conditions. Full-length PCR products were first sequenced directly, producing heterozygous sequences with degenerate positions labeled according to IUPAC nomenclature. Second, products were cloned prior to sequencing, using a Topo TA Cloning Kit (Invitrogen) following the manufacturers' recommendations. 2-8 positive colonies per plate were picked into 25 µL of ddH_2O, directly PCR amplified and sequenced in a polymorphic region of the gene (exon 2 resp. introns 1 and 5) to identify allelic variants. For each allele, 1-2 cloned products were sequenced to completion. Cloned products were compared to direct sequences in order to detect nucleotide misincorporations and to infer allelic phase. Up to 15 different combinations of nested primers were used to amplify shorter products of the seahorse MHIIb gene (300-2000 bp in length) to confirm homozygosity in individuals putatively homozygous for the full gene sequence. Samples were prepared for sequencing as described elsewhere (Bahr & Wilson 2011).

PCR reactions spanning three intronic single-bp repeat regions (dA and dT in intron 2, dC in intron 4) preferentially amplified one of the two alleles in 5 individuals, irrespective of PCR product length. Such a pattern may be caused by large differences in repeat length, where the shorter allele is preferentially amplified (Pompanon et al.

2005). The addition of either Betaine or 5% DMSO to PCR reactions in order to reduce secondary structure formation did not recover the second allele in these individuals. Primers flanking those repeat regions were therefore designed, and consecutive PCRs, excluding those single-bp repeats, allowed for the detection of heterozygous sequences in these individuals (see Supplementary Figure S1).

Processing of sequences

Sequence data were assembled using Sequencing Analysis 5.2 (Applied Biosystems), aligned with Muscle v.4.0 (Edgar 2004), and verified by eye in BioEdit v.7.0.9 (Hall 1999). Analyses on full-length exon sequences were conducted on uncropped sequences of 249 codons (exons 1-6, 747 bp). The complete dataset of exonic and intronic MHIIb sequences yielded 20 distinct alleles in our sample (see Suppl. Figure 1), but as single-bp repeat regions in intronic sequences could not be covered in all individuals (see above), the final dataset contains full length alleles from 14 out of 20 sequences (encompassing 14 different alleles, total length ≤ 3525 bp). In many cases, sequences identical in exon 2 (PBR) show intronic variability, suggesting either the evolutionary conservation of functional alleles with neutral variation accumulating over time, or the convergent evolution of PBR alleles. Full-length MHIIb sequences were assigned composite allele names, reflecting both exon 2 and full-length identity. The alleles Hiab-DAB*0101 and *0102, for example, have exon 2 sequences identical to the previously published Hiab-DAB*01 allele (Bahr & Wilson 2011), but differ in intronic sequence and are numbered consecutively.

Analyses of sequence polymorphism

DnaSP v.4.90.1 (Librado & Rozas 2009) was used to calculate standard diversity estimates. Intron diversity *d* (the average number of differences per site for intron sequences) was calculated using Mega v.4.0.2 (Tamura et al. 2007) under the Jukes-Cantor model. A sliding-window analysis of synonymous and non-synonymous exon variation (length 30, steps 12) was produced in SWAAP v.1.0.3 (Pride 2000) and CENSOR

was used for detection of transposons (Kohany et al. 2006). A CpG-island search was performed using EMBOSS CpGPlot/CpGReport (www.ebi.ac.uk/Tools/emboss/cpgplot/) (window size: 100 bp, step size: 1 bp, island length: 200 bp).

Positive selection

Mega v.4.0.2 was used to calculate dN and dS, as well as to test for positive selection in the dataset, applying a Z-test under a Jukes-Cantor model (10,000 permutations). Codons of the PBR were inferred through homology modeling to human HLA-DRB*01 alleles (Brown et al. 1993; Reche & Reinherz 2003). Site-specific positive selection was inferred using Codeml, implemented in the PAML v.4.2b package (Yang 2007).

Recombination / Gene conversion

We tested for recombination and gene conversion in the seahorse MHIIb gene using the default settings of RECCO v.0.93 (1,000 permutations) (Maydt & Lengauer 2006). RECCO reconstructs each sequence from the other sequences in the alignment using various scenarios of recombination and mutation, and the best fit model is inferred using a permutation test.

Results

Full-length MHIIb gene sequences were obtained for 10 seahorses collected from five populations (Supplementary Figure S1, GenBank ID.: xxx). The MHIIb gene of the seahorse consists of six exons (see Bahr & Wilson 2011 for gene map), with a total length of 3500 – 3532 bp and a total exon length of 747 bp. In high-quality DNA samples, we were able to amplify the complete gene using primers MHIIb-E1F and MHIIb-E6R. In several individuals, intronic repetitive elements inhibited amplification of single alleles, necessitating the sequencing of shorter gene fragments. In these animals,

the complete MHIIb sequence was obtained for one of the two alleles, while sequence data for the second allele near the repetitive regions of the introns are lacking (see methods, Suppl. Figure S1). MHIIb sequences comprise 20 different alleles due to intron variability and differences in repeat lengths, 14 of which provided complete, unambiguous sequences that were used to investigate intron diversity.

Sequence polymorphism in the PBR

The 9 MHIIb exon 2 sequences recovered here (Hiab-DAB*01, *04, *05, *08, *09, *11, *13, *16, and *17) are a subset of the 17 alleles identified in a larger sample of 101 individuals in a previous study (Bahr and Wilson 2011), and include 25 polymorphic nucleotide sites and 17 amino acid differences. Only 2 of the 25 variable sites identified are synonymous (dN = 0.050, dS = 0.012), leading to a strong signal of positive selection on exon 2 (Z-Test p = 0.010). The nucleotide diversity π of the seahorse exon 2 in this dataset is 0.040. Variable sites were identical between captive-bred individuals and samples from natural populations.

Sequence polymorphism outside the PBR

The 20 MHIIb alleles found in the seahorse include 12 distinct exonic alleles. Polymorphism outside the PBR of the seahorse is extremely low, with only two non-synonymous variants (located in exons 3 and 5) and a complete absence of synonymous substitutions (Fig. 1). A strong signal of positive selection over all exons is detected when the PBR is included (Z-Test, p = 0.003, Table 2), a signal which is absent when PBR sites are omitted from the analysis (Z-Test, p = 0.208, Table 2), despite the fact that the amino-acid substitution in exon 5 appears to be under site-specific positive selection (PAML p = 0.001). The nucleotide diversity π of exons of the seahorse MHIIb gene is 0.015, with an average number of 11 nucleotide differences between sequences. The amino acid sequence alignment of the complete MHIIb gene consists of 19 polymorphic sites and 12 distinct alleles (data not shown).

Complete intron sequences are available for 14 / 20 full-length MHIIb alleles. Length differences between these alleles (≤ 13 bp) reflect variation in repeat regions and single bp indels (Fig. 2, Suppl. Figure S1). Intron variability in the seahorse MHIIb gene is low (Fig. 3), with 1 – 12 variable nucleotide sites per intron for an indel-free alignment. The nucleotide diversity over all introns (p-distance ± SE = 0.004 ± 0.001, Table 2) is equivalent to the synonymous substitution rate over all exons (dS ± SE = 0.004 ± 0.003), but 3 times lower than dS in exon 2 (dS ± SE = 0.012 ± 0.010). Intron regions within 14 bp of exons (mean = 69 bp, range 14 – 177 bp) are completely conserved across all alleles.

Exons and introns differ in GC content. Exons are moderately GC-biased (0.45 AT vs. 0.55 GC), whereas introns have a strong AT bias (0.61 AT vs. 0.39 GC). This difference in nucleotide composition between exons and introns is statistically significant (Mann-Whitney U-Test: $n_1 = 12$, $n_2 = 14$, $U = 168$, $p < 0.001$). A high AT content in introns might, at least in part, be explained by the presence of AT-rich transposable elements (Chamary et al. 2006), and MHC genes are prone to the accumulation of transposons due to their high heterozygosity (van Oosterhout 2009). Fragments of 5 DNA-transposons were detected in the MHIIb sequences of *H. abdominalis* (transposon class: 4x hAT, 1x Mariner/Tc1), all of which have an AT content >50% (data not shown).

A comparison of nucleotide variability in 14 full-length MHIIb alleles shows the conservation of short sequence tracts between otherwise divergent sequences, leading to a patchwork pattern of nucleotide variation (Fig. 2). This pattern could be influenced by the significant levels of intralocus recombination inferred in our dataset (Recco p = 0.013), with an estimated number of 5 recombinant MHIIb full-length alleles. Recombination breakpoints were detected in the 5’ region of exon 2, in the 5’ to middle region of intron 2 and in the 5’ region of intron 4. Exon 3 of the seahorse MHIIb gene was detected as CpG-island (ratio observed : expected CG = 0.93), regions typically associated with high levels of recombination and/or gene conversion (Högstrand & Böhme 1999).

Discussion

The high variability of the peptide-binding b1-domain of the seahorse MHIIb gene, the region interacting with antigens, has been created and maintained by a combination of positive selection and intralocus recombination, consistent with findings from other vertebrates (see also Bahr & Wilson 2011). Strikingly, the remainder of the gene shows a complete absence of synonymous variation and extremely low levels of intronic diversity. Furthermore, short sequence tracts are conserved between otherwise divergent sequences of the seahorse MHIIb gene (Fig. 2), as expected under intralocus gene conversion.

Nucleotide diversity

Despite similarities between the level of non-synonymous variation in the PBR of the seahorse MHIIb and that of other teleosts, the nucleotide diversity of the full-length seahorse MHIIb gene is low ($\pi = 0.015$ over all exons incl. PBR, $d = 0.004$ over all introns; *Salmo salar*: $\pi = 0.026$ (Stet et al. 2002); *Leiocassis longirostris*: $\pi = 0.056$ (Shen et al. 2011); *Gasterosteus aculeatus*: $d = 0.016$ for intron 2 (Reusch & Langefors 2005); *Perca fluviatilis*: $d = 0.071$ for intron 1 (Michel et al. 2009)). We detected only two exonic substitutions (both nonsynonymous) outside the PBR. The absence of synonymous substitutions outside the b1-domain contrasts with previous studies where purifying selection has been shown to eliminate recessive deleterious mutations and contribute to an excess of synonymous relative to nonsynonymous substitutions outside the PBR (Hughes & Nei 1989). Unfortunately, almost all studies to date have focused exclusively on the peptide binding region of the teleost MH, and given the paucity of previous studies investigating complete MH genes in multiple individuals, it is unclear whether the low level of synonymous variation in the seahorse MHIIb gene reflects a unique evolutionary history in this group or whether it reflects a broader evolutionary pattern. A comparable study of coding DNA in *Salmo salar*, a species with a single MHIIb gene, revealed only 7 alleles in 84 specimen, with 1 synonymous and 2 non-synonymous

substitutions outside exon 2 (Stet et al. 2002), a pattern qualitatively similar to that recovered here. In contrast to this low diversity, Chinese longsnout catfish (*Leiocassis longirostris*), another species with a single gene copy, showed 7 synonymous and 11 nonsynonymous substitutions outside the PBR in 6 complete cDNA alleles (n = 5 specimens) (Shen et al. 2011). Klein et al. (1993) obtained two complete cDNA sequences that differed by 22 substitutions (9 synonymous and. 13 nonsynonymous) outside the PBR from a single *Aulonocara hansbaenschi* individual, a species with at least 2 MHIIb loci.

A reduced intron diversity relative to the synonymous variation detected in exon 2 has been observed in other teleosts (cyprinidae - intron 1 vs exon 2: Hughes 2000; threespined sticklebacks - exon 2 vs intron 2: Reusch & Langefors 2005; Eurasian perch - intron 1 vs exon 2: Michel et al. 2009). Consequently, the sharp contrast in the substitution patterns of the PBR and the remainder of the gene seems to be common for MHC genes, with the potbellied seahorse reflecting the lower limit of nucleotide diversity observed in other teleosts.

Molecular evolution

MHC genes have been shown to evolve under a combination of mutation, selection, recombination and gene conversion, gene duplication and/or loss, and drift. These mechanisms are responsible for the maintenance of contrasting patterns of nucleotide variation among different regions in MHC genes. The seahorse MHIIb gene shows a high number of nonsynonymous substitutions in the PBR, while genetic diversity outside this region is low, and variation is distributed in a patchwork fashion across the gene (Fig. 2).

The high PBR diversity of the seahorse MHIIb gene is maintained by positive selection on peptide binding sites, a pattern commonly observed in other studies (Yeager & Hughes 1999; Wegner 2008). A significant signature of intralocus recombination and/or gene conversion was detected in the seahorse MHIIb exon 2 (see also Bahr & Wilson 2011), a powerful mechanism which increases MHC allele number

and dN:dS ratio in combination with positive selection (Ohta 1997; 1998; 1999). A high dN:dS ratio may also be achieved by overdominant selection in combination with short term selection (Hughes & Nei 1989; Ohta 1998). Analyses of exon 2 in a large sample of individuals of *H. abdominalis* found no evidence of either an excess of MHIIb heterozygous individuals (HWE Exact Test: p = 0.08), or the non-random association of particular MHIIb alleles (see Bahr & Wilson 2011), suggesting that overdominant selection is unlikely to be the major force driving the high dN:dS ratio in this species.

Nucleotide diversity outside the PBR of the seahorse MHIIb gene is much lower than that detected in the b1-domain, a pattern typical of MHC genes (Yeager & Hughes 1999; Hughes 2000). However, in contrast to previous studies, the seahorse shows no synonymous substitutions outside the PBR. Intronic diversity (*d*) is also lower in the seahorse than that found in other teleosts, and shared polymorphism is found between otherwise divergent MHIIb alleles. The observed pattern of nucleotide variability in the seahorse MHIIb gene is thus congruent with expectations from intralocus gene conversion. Allelic gene conversion has been shown to reduce the allelic diversity of MHC genes (Ohta 1997; 1998; 1999). This form of conversion is also expected to lead to an excess of dS relative to *d*, as recombination across the exon-intron boundary homogenizes neutral MHC gene sequences. Following gene conversion, synonymous mutations linked to positively selected sites are maintained by hitchhiking, while sites outside the PBR are eliminated by random drift (Ohta 1998; 1999; Hughes 2000; Reusch & Langefors 2005). Intralocus gene conversion also has the potential to produce the observed patchwork pattern of shared polymorphism between alleles (Parham et al. 1995; Ohta 1999; Ohta 2000). The detection of several recombination breakpoints in the seahorse MHIIb gene, the lower nucleotide diversity in introns compared to exon 2, and the patchwork pattern of nucleotide diversity all indicate that intralocus gene conversion has a major influence on the molecular evolution of the seahorse MHIIb gene.

Evidence for the importance of recombination and gene conversion on MH genes has been found in other teleost species (sticklebacks: Reusch et al. 2004, Reusch & Langefors 2005; salmonids: Langefors et al. 2001, Aguilar & Garza 2007; perch: Michel

et al. 2009), but due to difficulties in the assignment of MH alleles to different loci, it has proven difficult to discriminate the relative importance of intra- and interlocus sequence exchange in such systems. Despite the use of a large number of primers in both conserved and variable regions of the MHIIb gene (Table 1), we found no evidence of additional gene copies and/or pseudogenes in the potbellied seahorse. The fact that only a single expressed copy of MHIIb was detected in a 454 sequencing screen (Bahr & Wilson 2011) supports the existence of a single functional copy of this gene in the seahorse. Despite the clear evidence for a single extant locus of MHIIb in the seahorse, interlocus gene conversion may have contributed to the present-day genetic diversity at this locus via a birth-and-death model of MHC evolution (Nei & Rooney 2005).

The deficit of synonymous variation found in the seahorse MHIIb gene could also be achieved if introns and synonymous sites are functionally constrained. Under this scenario, functionally important mutations assumed to be silent would be deleterious and would be eliminated by purifying selection (Hughes 2000; Chamary et al. 2006). Such functional constraints could include a biased synonymous codon usage to maximize translational efficiency, and/or to optimize mRNA stability, as well as conserved exon-intron junctions due to their role in splicing control (Hughes 2000; Chamary et al. 2006). Deviations from base composition parity may also effectively reduce dS and *d* (Wolfe et al. 1989; Hughes 2000). In fact, the seahorse MHIIb gene fits all these patterns and especially the biased GC-content, both at 3rd codon positions (76%) and in introns (39%), is consistent with expectations on functional constraints and on the relationship of GC content and neutral variation (Hughes & Nei 1989).

Conclusions

Intralocus gene conversion is thought to be an important process shaping molecular diversity via the exchange of genetic material among divergent alleles within genes. This mode of molecular evolution is expected to leave a prominent signature on genes experiencing balancing selection, producing a patchwork pattern of nucleotide substitutions and a heterogeneous distribution of neutral variation. Congruent with theoretical expectations for intralocus recombination, the seahorse MHIIb gene shows both characteristics. The high dN:dS ratio in the MHIIb PBR of the seahorse is typical of vertebrate MHC genes and has been influenced by a combination of positive selection and intralocus gene conversion and recombination. While recombination has been shown to effectively reduce intron diversity relative to exons in other species, this pattern may also be influenced by a biased nucleotide composition in the seahorse.

Supplementary Material

Supplementary Figure S1:

Nucleotide alignment of complete MHIIb sequences for 10 *H. abdominalis* individuals.

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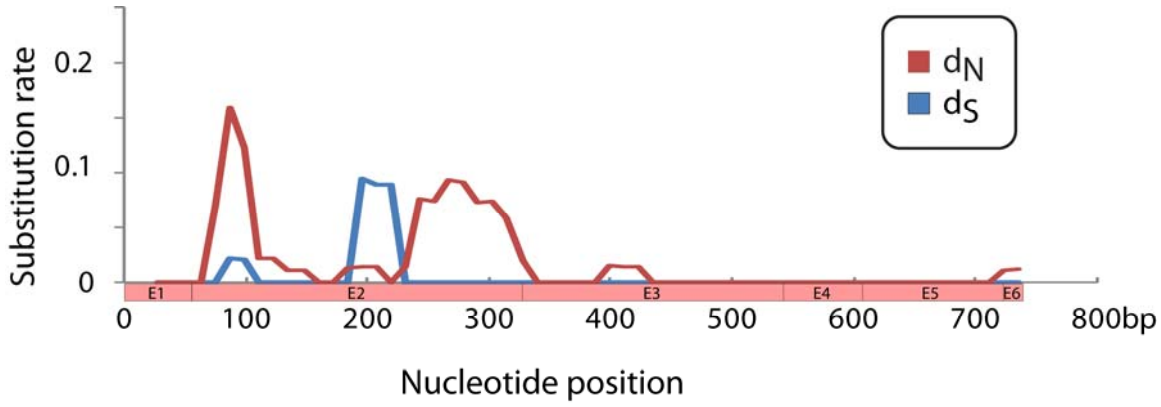
Table 1: Primers used to amplify and sequence MHIIb in *H. abdominalis.*

Name	Sequence 5'-3'	Location
MHIIb-UTR5F	CAAGTTTGTGCTCAGGTTGG	5'-UTR
MHIIb-E2F4	AACTCGAGTGACCAGAATGACATC	Exon 2
MHIIb-I2F	TAGGGCCTGACGAATATGGA	Intron 2
MHIIb-I2F4	AACGGAATCCATTTGGGAGT	Intron 2
MHIIb-I2R6	CAATGATTGTTCGGGTGTGA	Intron 2
MHIIb-E3F2	GCCTTACGTCAGACTTCACTCG	Exon 3
MHIIb-E3R3	GGCGTGTAGACCAGGTGAGA	Exon 3
MHIIb-E4F	GTGGAACACGCCAGCCTT	Exon 4
MHIIb-I4F2	TTTCCCACACGGTATCACAA	Intron 4
MHIIb-I4R	AACATCTCTCGGGTTGGTTG	Intron 4
MHIIb-I4R2	ACTTTACAGCAGGGGTCTTCA	Intron 4
MHIIb-E5F2	GCTGGACTGACTCTGGGTGT	Exon 5
MHIIb-UTR3R	ATCACTCAGTGCGAGCAGAA	3'-UTR

Table 2: Synonymous and non-synonymous substitution rates of full-length seahorse MHIIb alleles. Deviations from the null expectation of equality of dN to dS were tested with a Z-test (H_1 = positive selection, *<0.05, ns = not significant).

Locus	Length	N	Alleles	dN	dS	dN / dS	d
Exon 2	273	10	9	0.050	0.012	4.17*	
Exons 1-6	747	10	12	0.019	0.004	4.75*	
Exons 1-6 non-PBS	675	10	11	0.007	0.003	2.33^{ns}	
Introns	≤ 2775	10	14				0.004

Figure 1: Distribution of synonymous (dS) and non-synonymous (dN) variation across exons of 12 MHIIb alleles of the seahorse. A sliding-window analysis with a window-length of 30 and a step-length of 12 was used.



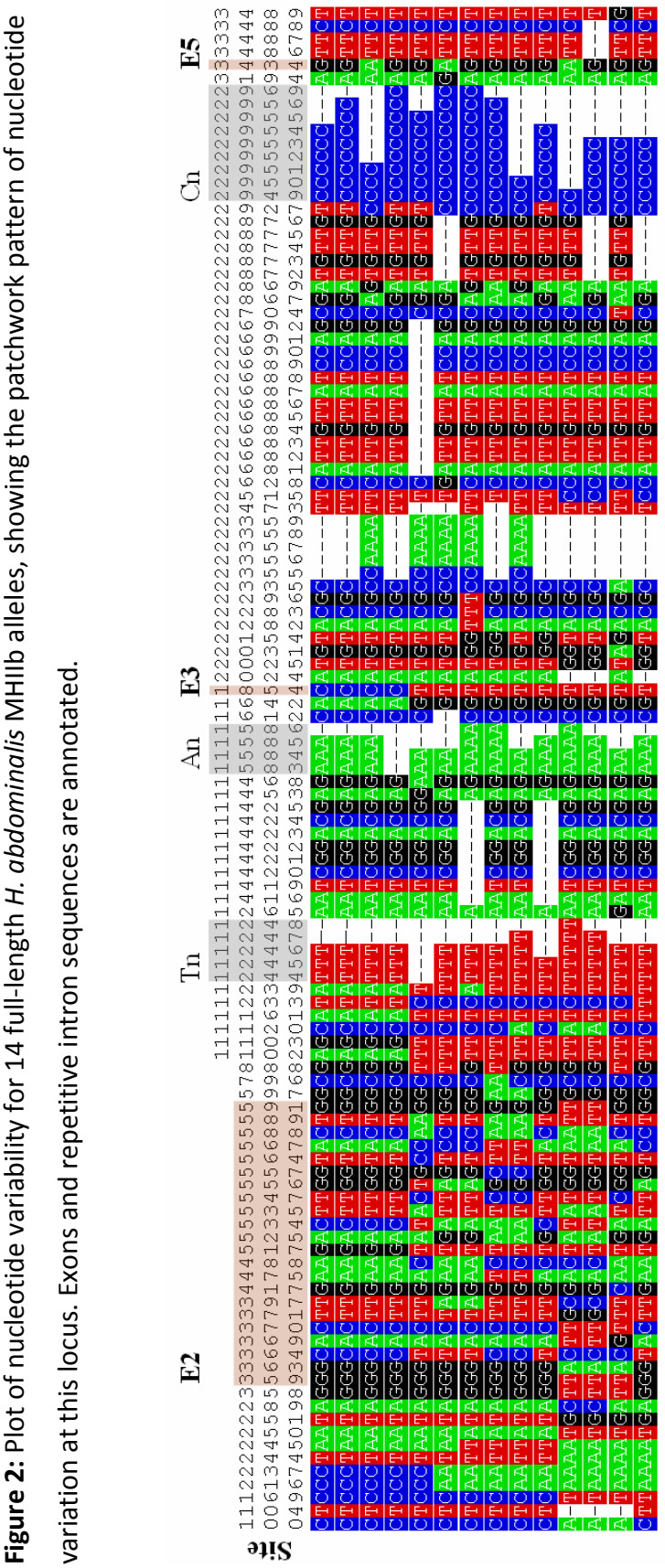


Figure 2: Plot of nucleotide variability for 14 full-length *H. abdominalis* MHIIb alleles, showing the patchwork pattern of nucleotide variation at this locus. Exons and repetitive intron sequences are annotated.

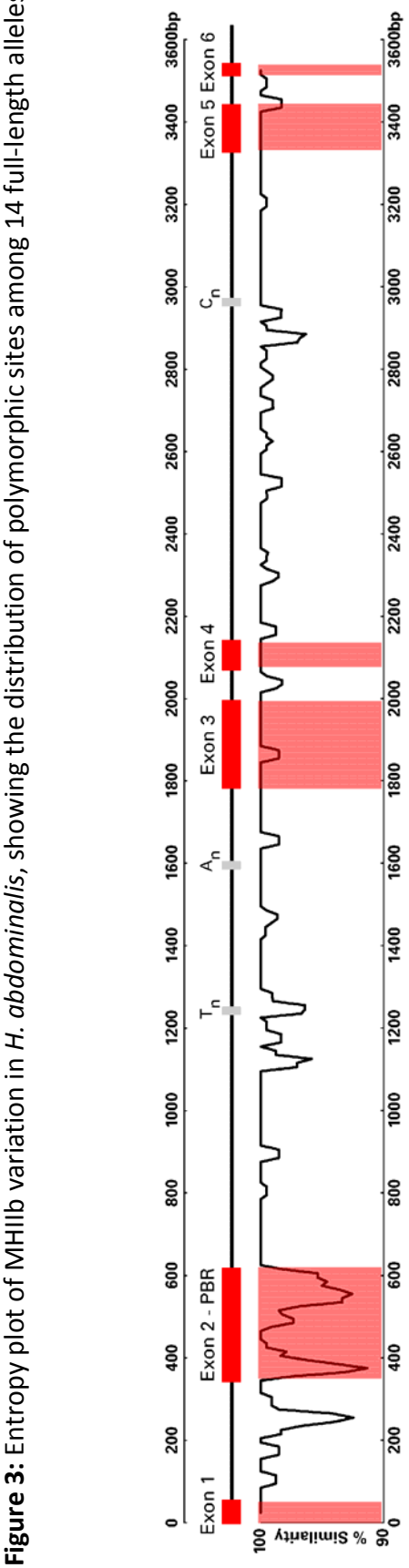
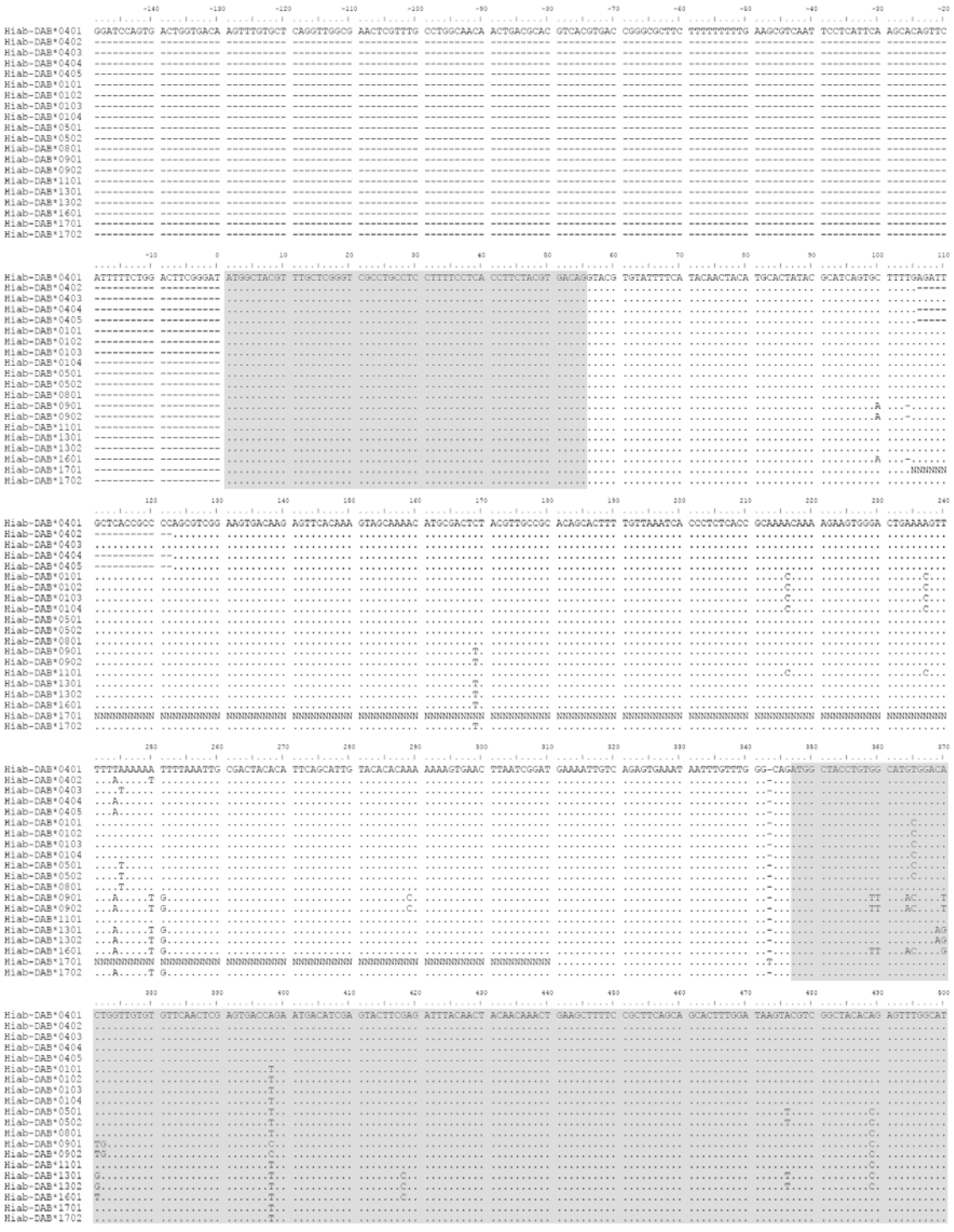
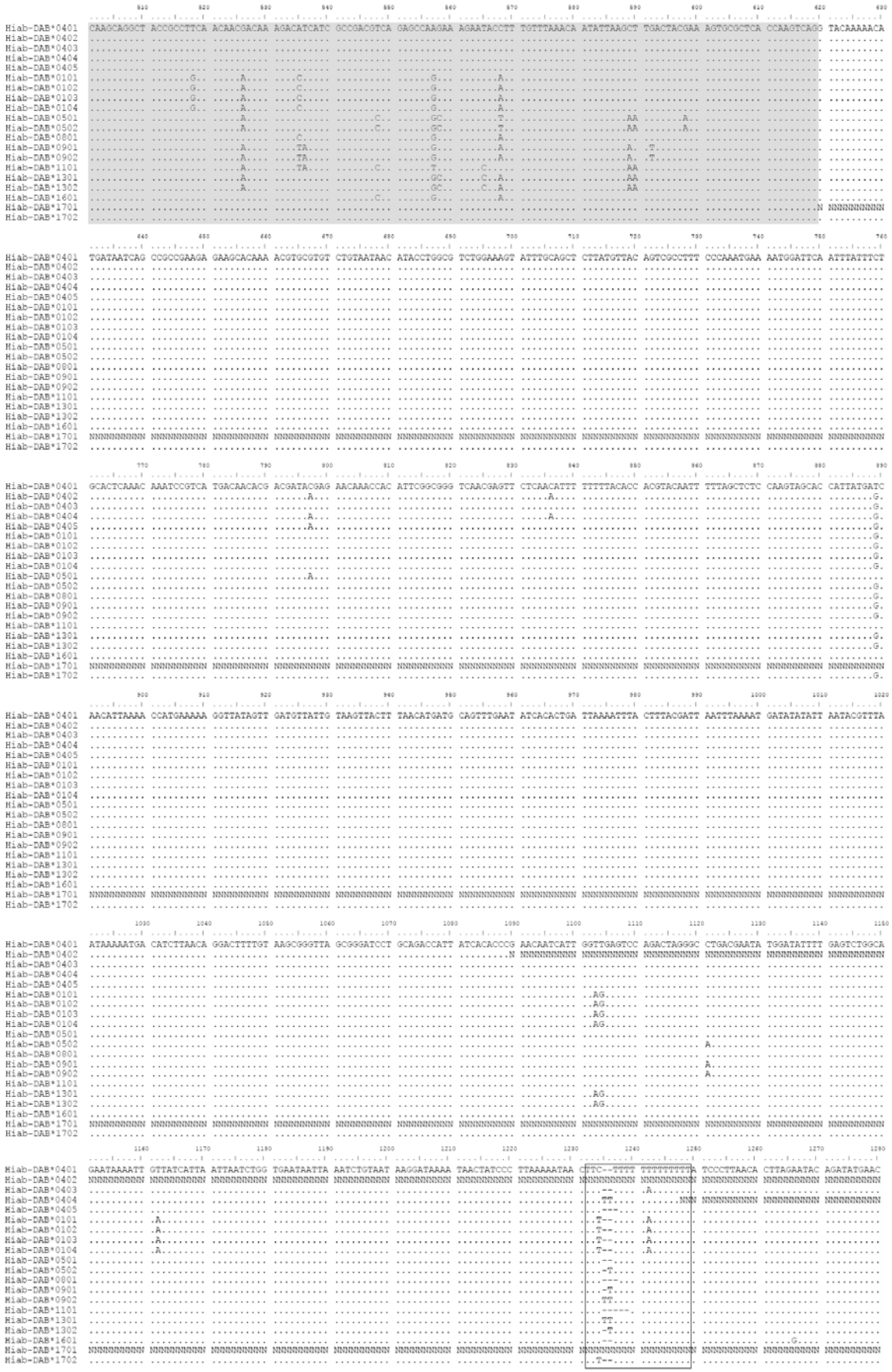


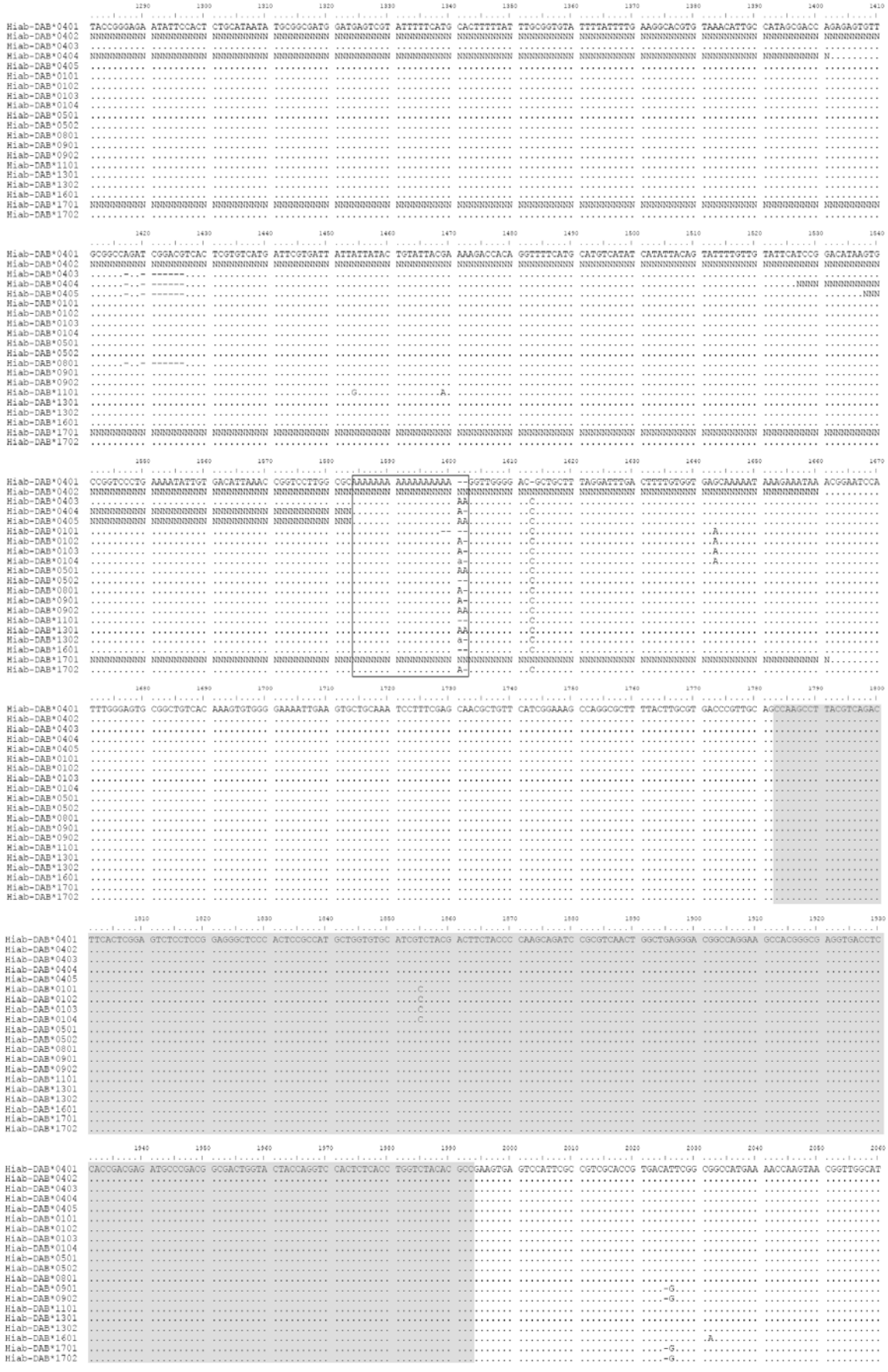
Figure 3: Entropy plot of MHIIb variation in *H. abdominalis*, showing the distribution of polymorphic sites among 14 full-length alleles.

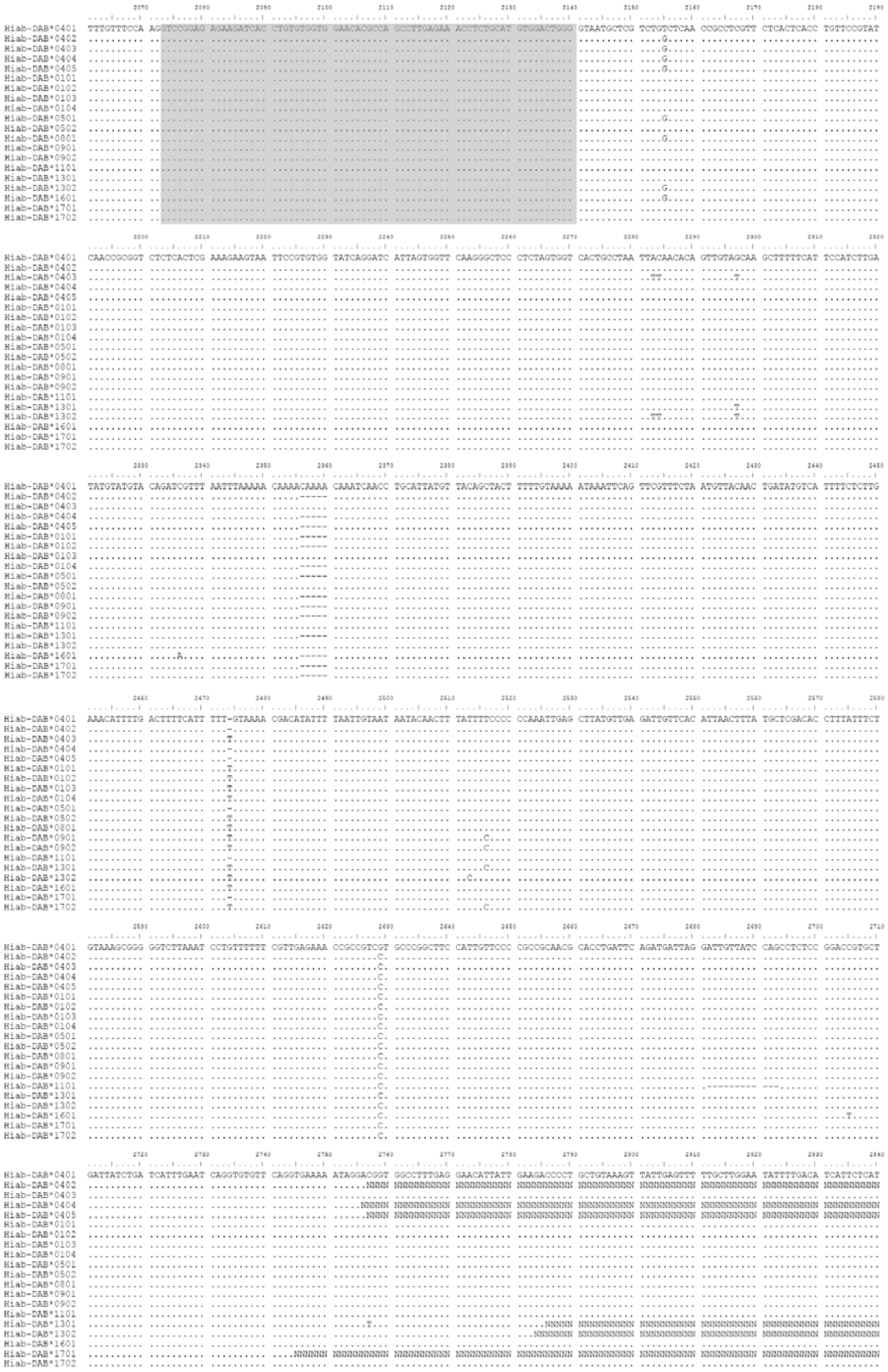
Supplementary Material

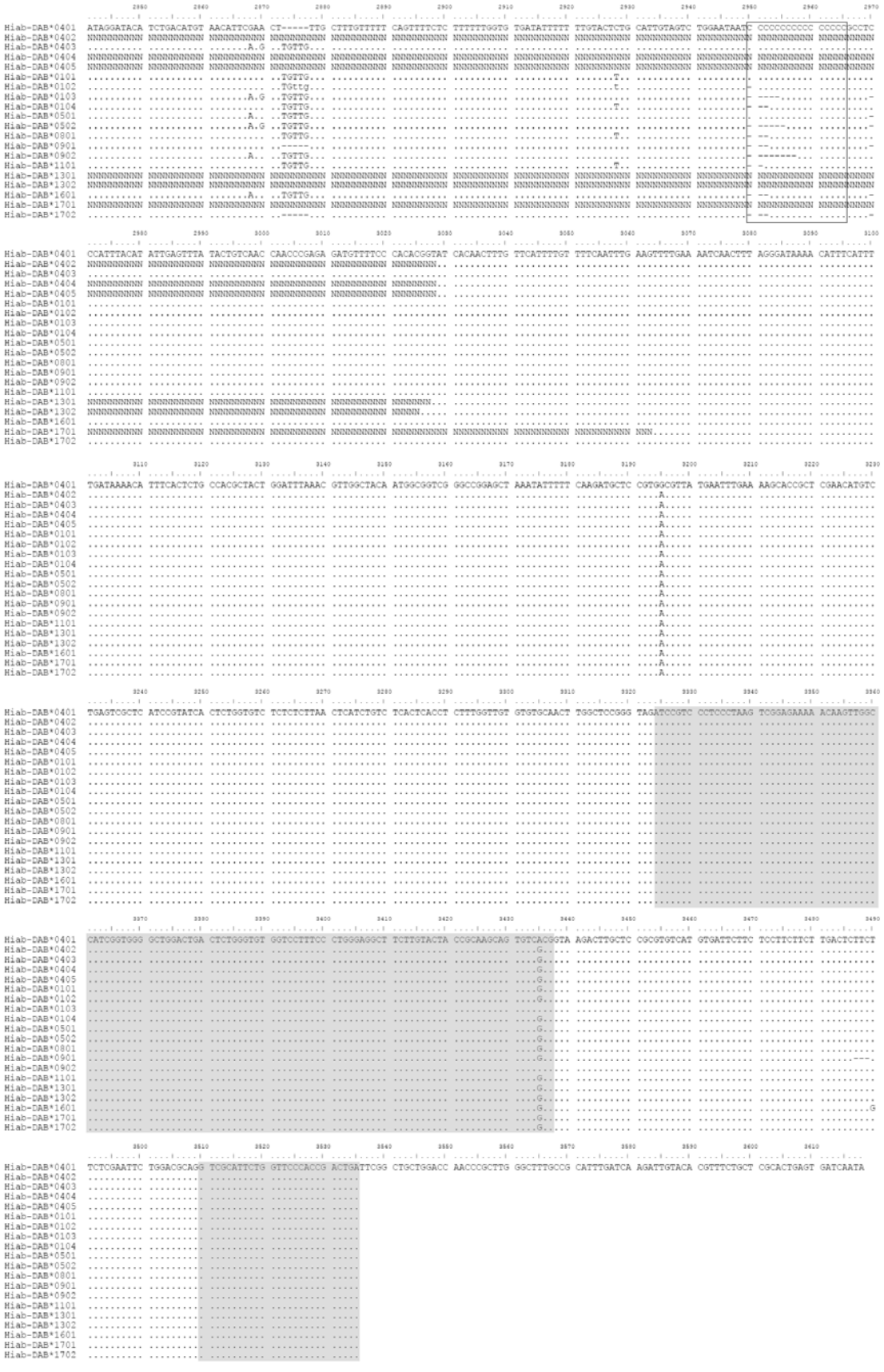
Supplementary Figure S1: Alignment of 20 complete MHIIb nucleotide sequences. Dots indicate positions identical to the first sequence. Exons are shaded in grey and intronic repetitive regions are highlighted with clear boxes. Hiab-DAB*0401 corresponds to Hiab-DAB*01, and Hiab-DAB*0402 to Hiab-DAB*02 from Bahr & Wilson (2011).











Mutual mate choice in the potbellied seahorse (*Hippocampus abdominalis*)

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Submitted to Behavioral Ecology

Abstract

Models of sexual selection have traditionally assumed dichotomous sex-roles, with one sex competing for access to mates, while the other sex is choosy. However, it is well known that mating decisions are realized by integrating information across multiple traits, the relative importance of which may be sex-specific. While a large body of work has investigated the influence of sexual signals on mating behavior, such traits have typically been studied in isolation, oversimplifying the multimodal communication associated with natural mating behavior. We investigated the impact of two key traits (MH class II beta-chain olfactory cues and body size) on mate choice decisions in the potbellied seahorse (*Hippocampus abdominalis*), a species considered to have female competition and male choice. We used a hierarchical experimental design (1. Olfactory cues only, 2. Olfactory and visual cues, 3. Free interaction) to investigate behavioral preferences and mating success of female and male seahorses under increasing levels of multimodal stimulation. Our data show that female seahorses prefer and mate with MHIIb-dissimilar males, while male seahorses mate randomly with respect to this trait. Conversely, males prefer and mate with large females, while females show no size-based mating preference. The multimodal integration of sex-specific mate preferences in mating behavior of the potbellied seahorse suggests the existence of mutual mate choice in this species. The results presented here suggest that more comprehensive studies of mating behavior, considering both female and male preferences for multiple traits, may lead to a more nuanced understanding of how sexual selection operates in natural populations.

Introduction

Mate choice decisions involve the integration of information provided via a wide range of behavioral, morphological, olfactory and vocal signals (Andersson 1994; Candolin 2003). The importance of such cues during mate choice depends not only on environmental conditions, but may also differ between the sexes (Andersson 1994; Houde 2001). Models of sexual selection typically assume that mate choice decisions are dictated by the choosy sex, ignoring the influence of mate preferences of the competitive sex on realized mating behavior (but see Bergstrom and Real 2000). This bias has been reinforced by the majority of behavioral studies, which tend to focus exclusively on the mating preferences of the presumptively choosy sex (typically the females) (e.g. Candolin 2003; but see Ahnesjö 2010).

Bony fishes are well suited for the study of sexual selection, due to their exceptional diversity, not only in terms of species numbers, but in reproductive patterns, parental care modes and mating patterns (reviewed in Amundsen 2003). When mating preferences of both male and female teleosts have been investigated under similar experimental conditions, sex-specific differences in trait preferences have often been detected. The most widely studied visual cues, for example, are body size and color patterns. The majority of studies on fish species incorporating both female and male preference experiments have found a preference of large mates by males, but not by females (e.g. *Nerophis ophidion* (Berglund et al. 1986), *Hippocampus abdominalis* (Mattle and Wilson 2009), *Gymnogobius isaza* (Morimoto et al. 2010)), while others have observed the opposite pattern (e.g. *Micropterus dolomieu* (Hanson and Cooke 2009), *Pomatoschistus minutus* (Kvarnemo and Forsgren 2000)), and/or preferences of large mates by both sexes (e.g. *Syngnathus typhle* (Berglund et al. 1986), *Poecilia reticulata* (Reynolds and Gross 1992; Herdman et al. 2004)). Size-based preferences are thought to reflect the fecundity benefits of mating with larger individuals and/or the competitive advantages of large body size. Preferences for more colorful or ornamented mates have also been detected in both males (e.g. *Nerophis ophidion* (Berglund et al.

1986), *Gobiusculus flavescens* (Amundsen and Forsgren 2001), *Syngnathus typhle* (Berglund and Rosenqvist 2001)) and females (e.g. *Gasterosteus aculeatus* (Bakker 1993)).

The genes of the major histocompatibility complex (MHC / MH in Actinopterygii) play an important role in determining individual odor (reviewed in Penn 2002). In addition to the importance of MHC genes as an integral part of the adaptive immune system of vertebrates, MHC-mediated odor cues have been shown to be important in mate choice, individual recognition and inbreeding avoidance (Penn and Potts 1999; Penn 2002; Boehm and Zufall 2006; Milinski 2006). According to the "divergent allele advantage" model, individuals with diverse MHC alleles are able to recognize a broader spectrum of pathogen-derived antigens than individuals carrying similar alleles (Wakeland et al. 1990; Sommer 2005). MHC-based mate choice may offer several advantages to the choosy individual (reviewed in Penn and Potts 1999): Firstly, parents may be able to actively enhance the immunocompetence of their offspring. Secondly, a moving target is provided during host-parasite-coevolution. Finally, inbreeding can be avoided by rejecting MHC-identical mates. As all three of these benefits can be realized by mating with MHC-dissimilar individuals, behavioral studies of MHC-based mating preferences have typically focused on disassortative mating in females and/or males. Olfactory and mating preferences for MHC-dissimilar mates have been detected in a wide range of vertebrate species (e.g. *Homo sapiens* (Wedekind et al. 1995; Wedekind and Furi 1997), *Mus* spp. (reviewed in Penn and Potts 1999), *Salmo salar* (Landry et al. 2001; Consuegra and de Leaniz 2008), *Oncorhynchus tshawytscha* (Neff et al. 2008), *Rhodeus ocellatus* (Agbali et al. 2010), but see: Paterson and Pemberton 1997, Huchard et al. 2010). At the same time, there is increasing evidence that an intermediate MHC-diversity may sometimes be preferred during mate choice, due to an interaction between MHC allele number and T-cell repertoire size (*G. aculeatus* (Aeschlimann et al. 2003; Eizaguirre et al. 2009), *Salmo trutta* (Forsberg et al. 2007)).

While it is clear that females of many fish species are capable of discriminating MH genes on the basis of olfactory cues, studies of male reproductive behaviour have failed to detect MH-based mating preferences (Forsberg et al. 2007; Neff et al. 2008).

Analyses of olfactory performance in mammals indicate that females typically outperform males in most olfactory measures (e.g. detection, sensitivity and discrimination) (reviewed in Good and Kopala 2006), and while comparable studies on fishes are rare, male and female teleosts often respond differently to olfactory cues (Lastein et al. 2006; Neff et al. 2008; Ratterman et al. 2009). Sex-specific differences in olfactory abilities might explain the lack of male MH-based mate choice observed in fishes. Alternatively, if preferences for MH cues are more pronounced in the choosy sex, the absence of male MH-based mate choice in this group could reflect the fact that all previous studies have investigated species with female choice.

As behavioral studies have continued to increase in their sophistication, incorporating multiple traits and the perspectives of both sexes, there is now clear evidence that both females and males show preferences, often on the basis of very different mating cues (Candolin 2003; Ahnesjö 2010). We investigated the effects of olfactory and visual cues on mate choice decisions in the potbellied seahorse *Hippocampus abdominalis*, focusing on MHIIb-mediated odor cues and body size, a sexually-selected morphological trait (Mattle and Wilson 2009). The seahorse shows a unique reproductive system with a highly developed form of male parental care. Eggs transferred by the female during mating are brooded in a pouch on the males' abdomen, a phenomenon termed 'male pregnancy' (Stölting and Wilson 2007). Previous studies on syngnathid fishes (seahorses and pipefishes) have focused on the role of phenotypic and environmental traits (e.g. body size, sex ratio) in sexual selection (e.g. Berglund et al. 2005; Mattle and Wilson 2009), but the role of olfactory cues has only recently been explored (Ratterman et al. 2009; Sundin et al. 2010; Lindqvist et al. 2011). Behavioral observations of natural populations of the potbellied seahorse *H. abdominalis* have shown evidence of female-female competition and male choice in this species (Wilson and Martin-Smith 2007), a widespread pattern in syngnathid fishes believed to be associated with the high levels of male parental care in this group (Wilson et al. 2003).

We used a hierarchical experimental design to investigate the role of olfactory and visual cues in mate choice decisions of male and female potbellied seahorses. We

first carried out a sequential preference experiment, providing focal individuals of both sexes with olfactory cues derived from individuals differing in their MHIIb-dissimilarity. A second preference experiment analyzed the combined effect of visual and olfactory cues on focal individual behavior. Finally, individual mating behavior was investigated in a large free-interaction experiment, which aimed to determine whether the sex-specific preferences observed in the first two experiments influence mating behavior under semi-natural conditions.

Materials and Methods

Sample population

The potbellied seahorse, *Hippocampus abdominalis*, is a temperate water marine species occurring in coastal habitats around New Zealand and south-eastern Australia. Seahorses are listed under Appendix II of the United Nations Convention on the International Trade in Endangered Species (CITES) and all individuals used in our mate choice experiments originate from a large captive-bred population (Seahorse Australia, Beauty Point) derived from individuals collected from three Tasmanian sites (Wilson and Martin-Smith 2007). This population has a high neutral genetic diversity, comparable to natural Tasmanian populations (see Suppl. Fig. 1 in Bahr and Wilson 2011). In August 2006 and 2008, 6-month old, sexually mature individuals were transferred to a large recirculating marine system at the University of Zurich, Switzerland, where they were kept segregated by sex prior to the experimental period to avoid potential pair bonding. Stock tanks were connected to a central seawater circulation system and contained artificial plants as holdfasts. Seahorses were fed with frozen *Artemia salina* and *Mysis relicta* ad libitum twice per day.

After 1 month of acclimatization to laboratory conditions, seahorses were anesthetized by placing them for 2 – 6 min in 10 l of a 55 ppm AQUI-S solution

(isoeugenol; AQUI-S, Lower Hutt, New Zealand) in 33 ppt salt water. Fin clips of anesthetized animals were collected for genetic analysis, and digital pictures of animals in lateral orientation were taken for standard length measurements. Individually-numbered plastic neck tags (1/8"x 1/4", FTF-69, Floy Tag, Seattle) were attached to seahorses using polyester thread to allow for individual identification. Tagging showed no effect on the behavior or health of the animals, which was monitored daily.

Microsatellite and MHIIβ genotyping

To infer genetic diversity and parentage, we extracted whole genomic DNA from fin clips (adults) or muscle tissue (offspring) using a DNeasy 96 Tissue Kit (QIAGEN), and genotyped individuals at 4 neutral microsatellite loci (Habd3, Habd6, Habd7, Habd9; Wilson and Martin-Smith 2007). Microsatellite PCR reactions were performed in a MJ DNA Engine Tetrad machine. Approximately 20 ng DNA was used in 10 µl reactions containing 1 µl 10x ThermoPol reaction buffer (NEB), 1 mM $MgCl_2$, 200 nM primers, 0.4 µM dNTPs (Roche) and 0.4 U Taq DNA polymerase (NEB). PCR running conditions included 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 30 min. For genotyping on an ABI 3730 automated sequencer (Applied Biosystems), PCR reactions were diluted 1:25 in ddH_2O. 1.5 - 2.0 µl of the diluted products from each of the independent microsatellite amplifications were combined in a plate containing 0.07 µl GeneScan 500 LIZ genotyping standard (Applied Biosystems), 9.93 µl HiDi Formamide (Sigma) and 4 µl ddH2O. Results were automatically scored using GENEMAPPER 4.0 (Applied Biosystems) and microsatellite alleles were verified by eye for each sample.

To test for MH-dependent mate choice in the potbellied seahorse, we assessed MH class II beta-chain gene diversity by sequencing the complete peptide binding region (PBR, exon 2, 273 bp) in all individuals (Bahr and Wilson 2011). Sequence data were aligned and verified by eye in BioEdit v.7.0.9 (Hall 1999). Allelic phase of heterozygote sequences was inferred using a Bayesian statistical method implemented in PHASE v.2.1 using the default program settings (Stephens and Donnelly 2003).

Variables measured

Individual neutral genetic diversity was calculated as described in Aparicio et al. (2007), weighting the contribution of each locus according to its allelic variability. These genetic data allowed the estimation of parentage for all 196 analyzed newborn seahorses using Cervus 3.0.3 (Kalinowski et al. 2007).

To assess MHIIb diversity, we calculated the number of amino acid differences between exon 2 alleles carried by each individual using Mega v.4.0.2 (Tamura et al. 2007). This variable was consequently termed “intra-individual MH-distance”, while the average allelic difference between individuals was recorded as “MHIIb-dissimilarity”. The MH-dissimilarity between male and female seahorses consequently reflects the average expected dissimilarity in their offspring (Landry et al. 2001; Neff et al. 2008).

We also investigated the effect of body size on behavioral preferences in experiments 2 and 3. The standard length of each seahorse was calculated with tpsDig v2.12 (Rohlf 2010) using digital pictures and arbitrary landmarks along the lateral body axis, from the tip of the snout to the tip of the tail.

Experiment 1 – Olfactory cues

Our first experiment was conducted daily between May and July 2009, investigating MH-based olfactory preferences in male and female seahorses using a sequential mate choice design. For each focal individual, 3 different olfactory stimuli, differing in their MHIIb-dissimilarity to the focal individual, were used. 25 focal females were used with 18 different male stimuli and 25 focal males with 17 different female stimuli.

We used 2 experimental tanks (55 x 55 x 75 cm, circa 210 l; Fig.1) located in the same room as stock tanks and connected to the same seawater circulation system. Choice tanks were divided into 3 sections (Fig. 1), including a neutral zone (half of the aquarium) and 2 preference zones (each ¼ of the aquarium). An artificial plant, identical to those in the stock tanks, was provided as a holdfast shelter next to the outflow in the

center of the neutral zone. The 2 preference zones were separated by a solid divider, preventing the exchange of water and chemical cues between them. Stimulus water came from a separate 40 l tank (maintained at a temperature identical to the test tank) into which the stimulus individual was acclimatized for 14-16h before the experimental trial. The focal individual was transferred to the test tank at the same time. A 40 l tank without a stimulus animal served as a control. Stimulus and control tanks were connected to test tanks via 2 mm diameter silicone tubing, and water flow (1 ml/sec) was initiated at the start of each behavioral trial. Test, stimulus and stock tanks were illuminated by overhead fluorescent lighting. Water parameters were kept constant throughout the experimental period (temperature 19.5±0.3°C, pH 8.5±0.3, salinity 33.9±0.6 ppt) and the light regime was set to 14:10 hours (light:dark). Animals were not fed during the settling period or trial.

Focal individuals were simultaneously presented with stimulus water flow from an individual of the opposite sex (preference zone P-S) and control water (preference zone P-C). Three independent trials for each of the focal individuals were used to test absolute preferences for 3 different stimuli differing in MH-dissimilarity. Focal individuals were re-tested after a period of at least 3 days (range: 3 - 56 days). The side of the compartment for the stimulus water, the grouping of individuals and experimental tank were randomized for each trial. The behavior of focal individuals was recorded using an overhead camera (ABUS, Wetter, Germany) throughout the experimental trials, which were conducted within 1h of the onset of artificial illumination, the time at which seahorses are reproductively most active (Mattle and Wilson 2009). After each trial, seahorses were held in stock tanks, separated into experimentally experienced and inexperienced animals.

Statistical analysis - Experiment 1

Preliminary experiments using color tracers demonstrated that stimulus water reached the neutral zone of the test tank within 5-7 minutes (data not shown). Consequently, the behavior of focal seahorses was analyzed for one hour (3600 sec),

starting 5 minutes after the onset of the water-flow. From video records, we scored the time the focal fish spent with its full head length in either preference zone. Preference was quantified as the relative proportion of time spent in the preference zone with stimulus water (P-S) compared to the total time spent in both preference zones (P-S + P-C). Consequently, a value above 0.5 indicates a preference for the stimulus. Preference can only be calculated if the focal animal enters at least one of the preference zones, and a large proportion of experimental trials were excluded (44% for females, 25% for males), due to the low activity of the focal seahorse, a common observation in this species (Mattle and Wilson 2009). Focal individuals with preference scores from at least 2 of 3 trials were included in the analysis (8 females, 18 males). For each of these individuals, preference scores for MH-similar and MH-dissimilar stimuli were then compared in order to quantify individual behavior (paired data structure). If a focal individual showed a preference in all 3 trials, the 2 trials with the most divergent stimuli were included in the analysis. MHIIb-dissimilarity values between the stimulus and focal individual, as well as the difference in MH-dissimilarity between stimuli are provided in Suppl. Table 1.

We tested the influence of MHIIb-dissimilarity on individual preferences, measured as a proportion ranging from 0 to 1 (see above). We also calculated the coefficient of determination, measured as the proportion of variation in preference scores explained by MHIIb-dissimilarity, by comparing the difference in preference scores for MH-dissimilar and MH-similar stimuli relative to their MHIIb-dissimilarity. MH-similar and dissimilar stimuli did not differ significantly between the sexes (Mann-Whitney U-Test: $U = 65.0$, $n_1 = 8$, $n_2 = 18$, $p = 0.696$; Suppl. Table 1). Preference scores were arcsine-transformed when analyzed with one sample t-tests. Nonparametric tests were used unless otherwise indicated, as even minor deviations from normality and homoscedasticity in small datasets can lower the power of parametric tests, increasing the Type I error rate (Erceg-Hurn and Mirosevich 2008). All statistical analyses were performed in SPSS 17.0 (Chicago, IL, USA), with the exception of power analyses on 2-tailed Wilcoxon Signed Rank Tests, which were conducted in G*Power 3.1.2 (Faul et al. 2007).

Experiment 2 – Olfactory and visual cues

In our second experiment, we tested the relative importance of olfactory and visual cues in individual preference. This experiment was originally designed to test body size preferences in *H. abdominalis* (Mattle and Wilson 2009), but the presence of both visual and olfactory cues in this experimental design allows for ad hoc tests of MH-based mate choice. In this study, 30 male and female seahorses were offered a dichotomous choice between a large and a small partner, without considering relative MH-dissimilarity. MH class II gene diversity of the individuals involved in this experiment was subsequently assessed as outlined above. The seahorses used here were a separate cohort of individuals introduced into the lab in August 2006.

Statistical analysis – Experiment 2

Trials were analyzed for MH-based mate choice if the 2 stimuli differed in their MHIIb-dissimilarity to the focal individual. As in experiment 1, a large number of focal seahorses showed low activity levels in this experiment, failing to enter either preference zone (females 48%, males 23%), yielding a total of 15 trials for focal females and 18 for males.

We tested for the impact of MHIIb-dissimilarity on individual preferences. Preference was calculated as for experiment 1 as the time spent with the MHIIb-dissimilar stimulus divided by the total time spent with both stimuli. As in experiment 1, MH-similar and dissimilar stimuli did not significantly differ between the sexes (Mann-Whitney U-Test: $U = 107.0$, $n_1 = 15$, $n_2 = 18$, $p = 0.310$; Suppl. Table 1). Preference scores were arcsine-transformed when analyzed with one sample t-tests. MHIIb-dissimilarity values between stimulus and focal individuals, as well as differences in MH-dissimilarity between stimuli are provided in Suppl. Table 1. The difference in MHIIb-dissimilarity between stimuli in experiment 2 was less than that for experiment 1 (experiment 1: mean difference in MHIIb-dissimilarity ± SD = 2.89 ± 1.71 amino acids; experiment 2: 2.09 ± 1.63 amino acids; Mann-Whitney U-Test: $p = 0.047$, Suppl. Table 1).

We also tested for body size-based preferences in this experiment, following Mattle and Wilson (2009). Focal female (21.0 ± 1.4 cm, mean ± SD) and male (20.4 ± 1.8 cm) seahorses did not significantly differ in size (Mann-Whitney U-Test: U = 122.0, n_1 = 15, n_2 = 18, p = 0.638). The average size difference between stimuli was significantly larger in female trials compared to that for male trials (females: mean standard length difference ± SD = 3.3 ± 0.6 cm; males: mean ± SD = 2.5 ± 0.5 cm; Mann-Whitney U-Test: U = 41.5, n_1 = 15, n_2 = 18, p = 0.001).

A Generalized Linear Model (GZLM) was used to test the non-additive effects of MHIIb-dissimilarity and body size on preference scores. In contrast to the previous analyses, in which preference was calculated for either the large or the MH-dissimilar stimulus, variables used in the GZLM were calculated independent of stimulus size and MH-dissimilarity. Preference was instead calculated for stimulus 1, the stimulus used in the left compartment of the experimental tank, who might be small or large, MH-similar or dissimilar. MH-dissimilarity and body size were calculated as difference between stimulus 1 and 2. We used a Poisson distribution and a natural log link function, with the time spent with stimulus 1 as the dependent variable and the natural logarithm of the time spent with both stimuli as an offset variable (Garson 2010). Sex was set as fixed factor, and size and MH difference were included as covariates. Chi-square statistics were calculated to evaluate model effects using a likelihood ratio test; all other settings were fixed at SPSS 17.0 defaults. We used a scale weight variable to correct for data over-dispersion (variance > mean) (Garson 2010).

Experiment 3 – Free interaction

In a third experiment, seahorses were allowed to freely interact and mate over a period of 4 months (February – June 2010), to investigate whether mate choice under semi-natural conditions was influenced by MH- and/or body size cues. We established a breeding population of 25 males and 25 females, randomly chosen from our captive-bred population. 36 of these individuals were derived from experiment 1, which had been carried out 9 months before. Seahorses were transferred to a 750 l aquarium

(73 x 69 x 148 cm), which was connected to the same seawater circulation system as stock tanks. Shelters and holdfasts were provided using rocks, pipes and plastic plants to create a complex habitat. Water parameters were kept constant at the same values as in experiment 1 and seahorses were fed frozen artemia and mysids twice per day. Seahorses have been shown to mate monogamously within broods (Kvarnemo et al. 2000; Wilson and Martin-Smith 2007), and we sampled an average of 5 offspring (range 1-14) from each clutch for genetic analysis of parentage. No males died during the experimental period, but 5 females died during the 4 month experiment. These females were significantly smaller than the surviving females (Mann-Whitney U-Test: U = 14.0, n_1 = 5, n_2 = 20, p = 0.014). While only one of these females reproduced during the trial period, we included all 5 females in subsequent analyses, as the majority of these individuals were available for mating for at least half of the experimental period. An analysis excluding these females produced similar results for MHIIb, but influenced our analysis of body size, due to the small size of these individuals (data not shown). While body size analyses conducted without these individuals were non-significant, males exhibited a pattern of size-based mate choice consistent with that observed in the full dataset (Wilcoxon Signed Ranks Test: n = 17, Z = -1.634, p = 0.102).

Statistical analysis – Experiment 3

Neutral genetic diversity was high in the experimental population (homozygosity: mean = 0.1, range 0 – 0.5; (as calculated in Huchard et al. 2010), leading to extremely low levels of relatedness (mean = 0.05, range 0 - 1). Consequently, no mate choice for unrelated or diverse partners could be detected (data not shown). Parentage of seahorse offspring was inferred using 4 neutral microsatellite loci, as described above.

Digital pictures of all adults were taken at the conclusion of the experiment. Male and female seahorses in our experimental population were similar in size (females: mean ± SD = 23.09 ± 1.91 cm, males: mean ± SD = 22.44 ± 1.92 cm; Mann-Whitney U-Test: U = 240.5, n_1 = 25, n_2 = 25, p = 0.162). To test for size-based mate

choice, we compared the median size difference between an individual and its mating partner(s) (size mate – size focal individual) relative to its median size difference to all individuals of the opposite sex (size potential mate – size focal individual). This second measure reflects the expected size difference under random mating.

The seahorses used in this experiment carried 16 of the 17 previously-characterized MHIIb-alleles (no Hiab-DAB-E2*07) (Bahr and Wilson 2011) plus 2 additional MH-alleles (GenBank: JN398459-460). 9 / 50 individuals (18%) were homozygous for the MHIIb gene. Male and female intra-individual MH-distance did not differ significantly (females: mean ± SD = 6.80 ± 3.15 amino acids, males: mean ± SD = 5.48 ± 4.28 amino acids; Mann-Whitney U-Test: $U = 271.5$, $n_1 = n_2 = 25$, $p = 0.421$). We used an individual-based approach to test for MH-based mate choice, comparing each individual's median MHIIb-dissimilarity with its mating partners to its median MHIIb-dissimilarity to all individuals of the opposite sex, the expected pattern of MHIIb-dissimilarity under random mating.

Finally, we tested whether mating success, which includes both mating probability (mated, unmated) and frequency (the number of matings), was influenced by intra-individual MH-distance and body size, using a Generalized Linear Model (GZLM). We used a multinomial distribution with a cumulative negative log-log link function, which is well suited for distributions with many low values (Garson 2010). Model choice was based on Akaike information criterion (AIC) values, with low AIC values indicating a better model-fit (Garson 2010). Initial analyses included multinomial, negative binomial and tweedie distributions with different link functions. We used mating success (0-5 matings) as the dependent variable, sex as a fixed factor, and MH-distance and body size as covariates. Chi-square statistics were calculated using a likelihood ratio test to evaluate model effects.

Results

Experiment 1 – Olfactory cues

Are MHIIb-dissimilar stimuli preferred compared to MH-similar individuals?

Female seahorses showed significantly stronger preferences for MH-dissimilar stimuli than for MH-similar cues (preference(similar): median = 0.07, preference(dissimilar): median = 0.88; Wilcoxon Signed Ranks Test: n = 8, Z = -2.201, p = 0.028; Fig. 2a), while males showed similar preferences for MH-similar and dissimilar stimuli (preference (similar): median = 0.48, preference(dissimilar): median = 0.54; Wilcoxon Signed Ranks Test: n = 18, Z = -0.131, p = 0.896; Fig. 2b). The power to detect the difference in female preference scores was high (power = 0.88), despite the small sample size. The coefficient of determination (r^2) was estimated as 0.270 for females and 0.062 for males, indicating that MHIIb-dissimilarity explains a higher proportion of variation in female preferences than that for males.

Is the stimulus zone preferred compared to the control zone?

A comparison of the time spent in the stimulus zone relative to the total time spent in both preference zones (P-S + P-C), indicated that females avoided the smell of MHIIb-similar stimuli (One sample t-test: $t_7 = -3.663$, $p = 0.008$; Fig. 2a), but did not show a preference for MHIIb-dissimilar stimuli compared to the control (P-C) ($t_7 = 0.935$, $p = 0.381$), a pattern influenced by 2 individuals who did not enter the stimulus zone (P-S) during their trials (Fig. 2a). After excluding these individuals, a significant preference for MH-dissimilar stimuli was also detected ($t_5 = 6.274$, $p = 0.002$). In contrast, males showed no preference for either preference zone (female stimuli vs. control) (MH-similar trials: $t_{17} = 0.319$, $p = 0.754$; MH-dissimilar trials: $t_{17} = 0.400$, $p = 0.694$; Fig. 2b).

Experiment 2 – Olfactory and visual cues

Individuals likely evaluate multiple cues when making mate choice decisions (Candolin 2003). In experiment 2, focal individuals were simultaneously presented with both olfactory and visual cues from stimulus animals. 15 female and 18 male trials were included, after excluding trials in which both stimulus individuals had identical MHIIb dissimilarity values.

Do MHIIb-dissimilarity and/or body size influence preference?

A Generalized Linear Model for preference, including MHIIb dissimilarity, standard length and sex as main effects (AIC = 36.15), fit the data significantly better than an intercept-only model (omnibus test: LR chi^2 = 10.96, df = 3, p = 0.012). The addition of 2-way interactions (AIC = 40.92) did not improve the fit of the model (LR test, main-effects vs. main-effects + interactions: chi^2 = 1.224, df = 3, p = 0.747). None of these interactions explained a significant proportion of the variance in preference (sex * MH-difference: p = 0.960; sex * size-difference: p = 0.938; MH-difference * size-difference: p = 0.289). The main-effects model shows a significant positive effect of body size, but, in contrast to the results of experiment 1, no effect of MHIIb-dissimilarity on individual preferences. Model effects, along with parameter estimates, are provided in Table 1.

Are larger individuals preferred?

Female seahorses showed no preference with respect to body size (preference (large): 0.60 ± 0.38; One sample t-test (H_0 = 0.5): t_{14} = 0.969, p = 0.349), whereas males showed a significant preference for large females relative to small individuals (preference (large): 0.71 ± 0.27; t_{17} = 3.286, p = 0.004).

Are MHIIb-dissimilar stimuli preferred?

As in the GZLM, neither female nor male seahorses showed a significant preference for MH-dissimilar stimuli relative to similar individuals (females - preference (dissimilar): 0.49 ± 0.40, One sample t-test ($H_0 = 0.5$): $t_{14} = -0.254$, $p = 0.803$; males – preference (dissimilar): 0.58 ± 0.33, $t_{17} = 1.066$, $p = 0.301$).

Experiment 3 – Free interaction

In a third experiment, we tested whether the preferences inferred in the first 2 experiments influence mate choice decisions under semi-natural conditions. Over the course of the 4 month experiment, 38 matings were detected via genetic analysis of parentage, only 2 of which were from the same 2 parents, leading to 36 different mother-father pairs. A total of 15 / 25 females (60%) and 17 / 25 males (68%) reproduced during this period (Figs. 3, 4). Mating success of females ranged from 0 to 5 (1.52 ± 1.58, mean ± SD), while male mating success varied from 0 to 4 matings (1.52 ± 1.33) (Figs. 3, 4). Females had up to 4 different male partners and males had a maximum of 3 different female partners. Sex-specific variance in mating success did not differ significantly (variance females = 2.51, males = 1.76; Levene's test: $W_{1,48} = 0.677$, $p = 0.415$). Males released broods over as many as 4 consecutive days. Inter-brood intervals were ≥ 13 days for females and ≥ 25 days for males, indicating a higher potential reproductive rate (measured as the maximum number of offspring that can be produced per time unit) of females under experimental conditions, in contrast to the pattern observed in other seahorse species (Vincent 1992; Masonjones and Lewis 2000). Our data confirm within-brood monogamy in *H. abdominalis* (Wilson and Martin-Smith 2007) and between-brood polygamy within a breeding season (Woods 2000), even under the high density experimental conditions of our experiment.

Is mating random with respect to MHIIb-dissimilarity?

Females tended to mate more often with MHIIb-dissimilar males than expected by chance (Wilcoxon Signed Ranks Test: n = 15, Z = -1.822, p = 0.068, Fig. 3a), while males mated randomly with respect to MHIIb-dissimilarity, consistent with the results of experiments 1 and 2 (n = 17, Z = -0.630, p = 0.529, Fig. 3b).

Is mating random with respect to body size?

Males mated significantly more often with large females than expected under random mating (Wilcoxon Signed Ranks Test: n = 17, Z = -2.898, p = 0.004; Fig. 4a), consistent with the results of experiment 2, whereas female seahorses showed no pattern of size-based mate choice (Wilcoxon Signed Ranks Test: n = 15, Z = -0.398, p = 0.691; Fig. 4b).

Is mating success influenced by MHC and body size?

We were also interested to see if individual mating success is influenced by size and/or intra-individual MH-distance. A Generalized Linear Model using mating success as the dependent variable with main effects and 2-way interactions explained the data significantly better than the null model (Omnibus test: LR chi^2 = 25.36, df = 6, p < 0.001). Model effects, along with parameter estimates, are provided in Table 2. The fitted model shows a significant interaction between sex and intra-individual MH-distance (Table 2), indicating that the importance of individual MH-diversity on mating success differs between male and female seahorses. Sex-specific effects of body size were not significant in this analysis (p = 0.091, Table 2).

Female mating success decreased significantly with increasing intra-individual MH-distance (2-tailed Spearman's correlation: n = 25, r = -0.400, p = 0.048), while male mating success increased significantly with MH-distance (n = 25, r = 0.460, p = 0.021). Intra-individual MH-distance was significantly higher in males carrying rare allelic

variants (Spearman's correlation: MH-distance vs. population frequency of the rarest allele in each individual – males: r = -0.446, p = 0.025; females: r = 0.084, p = 0.689).

Mating success of female seahorses was positively correlated with body size (2-tailed Spearman's correlation: n = 25, r = 0.412, p = 0.041). Mating success of males, in contrast, was unrelated to size (2-tailed Spearman's correlation: n = 25, r = 0.019, p = 0.928).

Discussion

Using a hierarchical experimental design, we have shown that male and female seahorses differ in their preferences for morphological and olfactory traits. Despite these differences, mate choice decisions in the potbellied seahorse appear to be influenced by both sexes, with females mating with MHIIb-dissimilar partners and males mating more frequently with large-bodied females.

Female mate choice for MH

Female seahorses prefer MHIIb-dissimilar males over MH-similar individuals when presented with olfactory cues, a preference which is also evident in realized mating behavior. In contrast, we found no evidence of MH-based preferences in male seahorses, despite evidence of male mate choice in this species (Wilson and Martin-Smith 2007). Our results suggest that female-mediated sexual selection on MH genes in *H. abdominalis* likely contributes to the high MHIIb diversity observed in this species (Bahr and Wilson 2011). Consistent with theoretical expectations (Wakeland et al. 1990), males carrying divergent MHIIb alleles had higher mating success, a pattern which could reflect female preference for males carrying diverse and/or rare MHIIb alleles, or condition-related benefits associated with these alleles. Surprisingly, females carrying divergent MHIIb variants had lower than expected mating success, a counterintuitive pattern which requires further investigation. Overall, these results are

consistent with previous studies on teleosts, which indicate that females, but not males, use MH-based olfactory cues during mate choice (Neff et al. 2008; Forsberg et al. 2007).

While the results of experiments 1 and 3 support female-based preferences for MHIIb in the seahorse, no MH-based preferences were detected in experiment 2, where olfactory and visual cues were presented together. The results might reflect differences in the design of experiment 2, which was aimed at studying the effects of body size and included stimulus animals whose MHIIb-dissimilarity was more similar than in experiment 1 (see methods). While a divider separated odors produced by the stimulus animals, this divider did not extend into the choice compartment (Fig. 1 in Mattle and Wilson 2009), something which may have affected the focal individual's ability to distinguish odor cues. A follow-up experiment, using a similar tank design to that used in experiment 1, would be worthwhile to explore the significance of these results.

Male seahorses showed no evidence of MHIIb preference in any of our three experiments. Indeed, males showed no obvious preference for females on the basis of olfactory cues (experiment 1), suggesting that male *H. abdominalis* cannot detect, or do not use, such cues. A lack of male-based olfactory discrimination has also been observed in a close relative of the seahorse, the sex-role reversed *Syngnathus typhle* (Sundin et al. 2010; Lindqvist et al. 2011; but see Ratterman et al. 2009 on *Syngnathus scovelli*). Further investigations of male olfactory preferences in other sex-role reversed species would be beneficial in order to determine the conditions under which they use olfactory signals. Olfactory cues might be less important for males than visual signals, if fecundity selection favors males who mate with large females.

To our knowledge, evidence of male olfactory preferences for MH-based odor cues is restricted to a small number of studies on humans and mice (Wedekind and Furi 1997; Penn and Potts 1999), and has never been detected in any fish species. While such a pattern may reflect differences in the olfactory capabilities of males and females, this may also stem from the sex-specific production of MHIIb odor cues in this group. Recent work on *Gasterosteus aculeatus* suggests that a MH-independent signal essential for MH detection is produced exclusively by males in this species, effectively preventing

the detection of MH cues produced by females (Milinski et al. 2010). While the Milinski et al. (2010) study suffers from several methodological limitations, it suggests one mechanism by which MH-associated odors might be detected and used in a sex-specific fashion.

Male mate choice for body size

The results of our free interaction experiment indicate that male preferences for large-bodied females (experiment 2: Mattle and Wilson 2009) are realized under semi-natural conditions. Males mated more frequently with large females than expected by chance, whereas females showed no size-based mating pattern, suggesting that sexual selection may act more strongly on female than male body size in this species. Female mating success increased with body size, likely reflecting the combined effects of male size-based preferences, increased potential reproductive rate (Vincent 1990; Woods 2007), and/or the competitive benefits of large-bodied females. These results might help to explain the observation of female-biased sexual size dimorphism in natural populations of *H. abdominalis* (Martin-Smith and Vincent 2005; Wilson and Martin-Smith 2007). An investigation of a natural population of the Western Australian seahorse *H. subelongatus* found that mated females were significantly larger than unmated individuals, but detected no evidence of differential mating with respect to size in males (Kvarnemo et al. 2007), a result consistent with that observed for *H. abdominalis* in our study.

Mutual mate choice

The potbellied seahorse is considered to be sex-role reversed based on observations of strong female-female competition and male choice in natural populations of this species (Wilson and Martin-Smith 2007). Our study suggests that both sexes actively influence mate choice decisions in *H. abdominalis*, with sexual selection simultaneously favoring large females and MH-dissimilar males, a pattern of mutual mate choice based on sex-specific mating cues. These results highlight how the

preferences of both sexes may influence mate choice behavior, and call into question the dominant dichotomous view of a choosy and a competitive sex (see also Berglund et al. 2005; Ahnesjö 2010). Theoretical models predict the evolution of mutual mate choice in species in which relative parental investment is comparable between the sexes (Kokko and Johnstone 2002), results supported by recent empirical studies that show that mutual mate choice occurs more frequently than previously expected, especially in monogamous species where both parents make a considerable investment in reproduction (see Appendix Table 1 in Hooper and Miller 2008; South and Arnqvist 2011).

Conclusions

Using a combination of mate choice experiments and observations of matings in a free-interaction mating arena, we have demonstrated that male and female seahorses show distinct mating preferences, both of which are realized under semi-natural conditions. MH-diversity influences female mate choice and male mating success in the seahorse, while male mate choice and female mating success are dependent on body size, leading to mutual mate choice in this species.

Our study demonstrates how the integration of multiple mating cues and both male and female perspectives in behavioral research can result in a more nuanced appreciation of mate choice and sexual selection. Studies such as this challenge the traditional dichotomous view of the sexes in terms of sex-roles, with a choosy and a competitive sex, and call into question models of sexual selection based on such assumptions. We have provided important evidence for how sex-specific preferences for two key traits may influence reproductive outcomes, but these are only two of what are likely a large number of traits influencing mate choice decisions in this system. Future work should investigate the multivariate interactions between such traits and, at the same time, explore the implications of mutual mate choice and multimodal integration on standard models of sexual selection.

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Table 1

Experiment 2 - Generalized Linear Model results for the effects of MHIIb-dissimilarity and body size on male (n = 18) and female (n = 15) preferences in *H. abdominalis*.

	Model effects		Parameter estimates	
Factor	**LR chi^2**	**p-value**	**B**	**odds ratio**
Sex	0.005	0.943	0.019	1.019
Stimuli - Difference in body size	5.815	0.016	0.110	1.116
Stimuli - Difference in MH-dissimilarity	1.612	0.204	0.066	1.068

LR chi^2 = likelihood ratio chi-square

Table 2

Experiment 3 - Generalized Linear Model results for the effects of individual MH-distance and body size on mating success in *H. abdominalis* (n = 50).

	Model effects		Parameter estimates	
Factor	**LR chi^2**	**p-value**	**B**	**odds ratio**
Sex	1.618	0.203	4.912	135.860
Body size	0.107	0.743	0.071	1.074
MH-distance	1.101	0.294	-0.815	0.443
Sex * Body size	2.851	0.091	-0.286	0.751
Sex * MH-distance	14.503	< 0.001	0.296	1.344
Body size * MH-distance	1.264	0.261	0.031	1.032

LR chi^2 = likelihood ratio chi-square

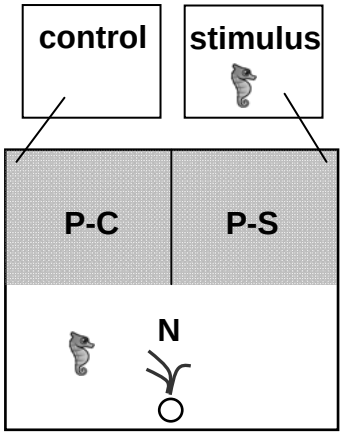

Figure 1

Experimental design of experiment 1, showing preference zones (stimulus P-S, control P-C), the neutral zone (N), as well as locations of the water outflow (circle) and artificial holdfast.

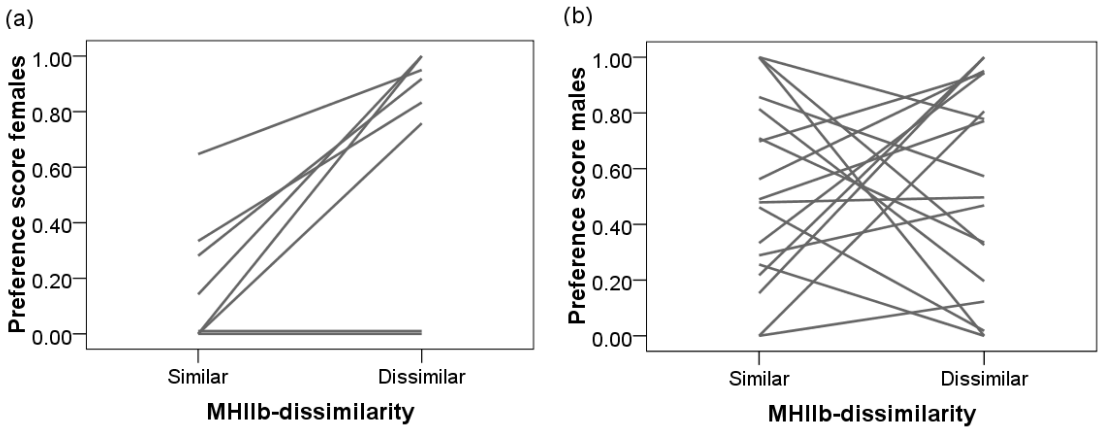

Figure 2

Experiment 1 - Preference scores of (a) female (n = 8) and (b) male (n = 18) seahorses for MHIIb-similar and dissimilar stimuli. A score of 0.5 indicates that the focal individual spent equal time on the stimulus and control sides of the tank, whereas scores <0.5 show a preference for the control and scores >0.5 indicate a preference for the stimulus.

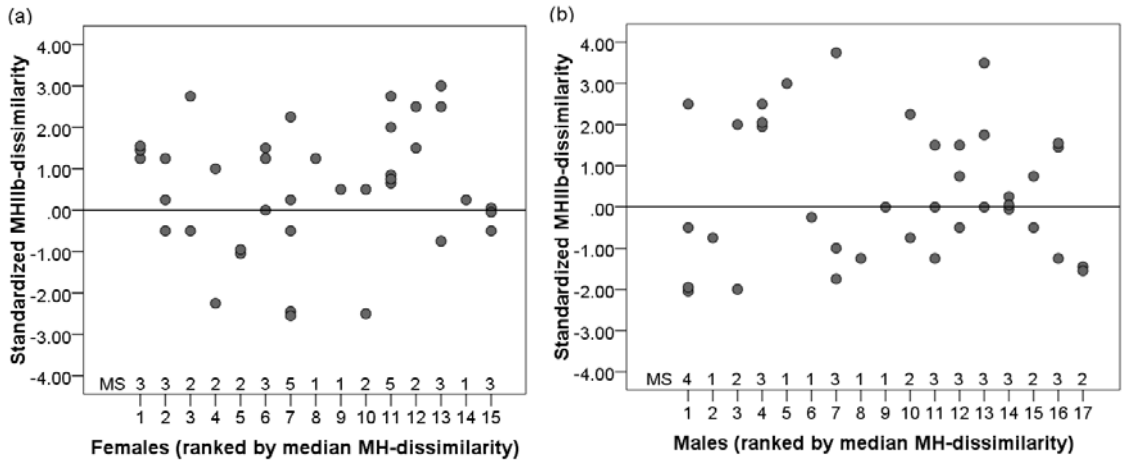

Figure 3

Experiment 3 - Standardized MHIIb-dissimilarities of (a) female and (b) male seahorses during mating. Standardized MH-dissimilarity = MH-dissimilarity of a mating event – median MH-dissimilarity to all available mates (expectation under random mating). Scores > 0 represent a greater MH-dissimilarity to a mate than expected by chance, while scores < 0 indicate a lower than expected MH-dissimilarity. MS = mating success

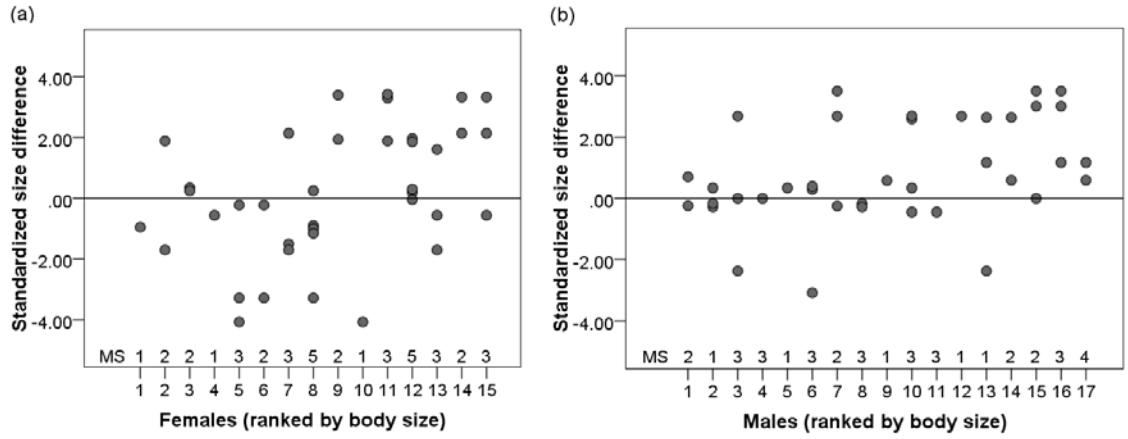

Figure 4

Experiment 3 - Size of mated (a) female and (b) male seahorses. Standardized size difference = (size mate - size focal individual) - median (sizes of all available mates - size focal individual). Scores > 0 represent matings with larger individuals, while scores < 0 reflect matings with smaller than expected individuals. MS = mating success

Supplementary Table 1
MHIIb-dissimilarity of stimulus seahorses. Sex is provided for focal individuals.
Exp = experiment, F = Female, M = Male, N = Sample size, MWU = Mann-Whitney U

Exp	Sex	Stimuli	N	Mean ± SD	Range	Statistics (F : M)	Statistics (exp1 : 2)
1	F	Similar vs dissimilar	8	2.59 ± 1.21	1.00 – 4.50	**MWU-Test:** U = 65.0	
1	M	Similar vs dissimilar	18	3.01 ± 1.91	0.25 – 7.25	$n_1 = 8$, $n_2 = 18$ p = 0.696	**MWU-Test:** U = 299.5
2	F	Similar vs dissimilar	15	1.75 ± 1.29	0.25 – 4.25	**MWU-Test:** U = 107.0	$n_1 = 26$, $n_2 = 33$ p = 0.047
2	M	Similar vs dissimilar	18	2.38 ± 1.85	0.25 – 7.75	$n_1 = 15$, $n_2 = 18$ p = 0.310	
1	F	Similar	8	5.47 ± 1.69	3.50 – 8.00	**MWU-Test:** U = 65.5	
1	M	Similar	18	5.13 ± 1.25	2.00 – 6.75	$n_1 = 8$, $n_2 = 18$ p = 0.717	
1	F	Dissimilar	8	8.06 ± 1.02	6.75 – 9.25	**MWU-Test:** U = 67.5	
1	M	Dissimilar	18	8.03 ± 1.48	5.25 – 10.00	$n_1 = 8$, $n_2 = 18$ p = 0.800	
2	F	Similar	15	5.07 ± 1.73	2.00 – 8.50	**MWU-Test:** U = 123.0	
2	M	Similar	18	4.69 ± 1.76	1.00 – 7.75	$n_1 = 15$, $n_2 = 18$ p = 0.663	
2	F	Dissimilar	15	6.82 ± 1.86	3.50 – 10.00	**MWU-Test:** U = 123.5	
2	M	Dissimilar	18	7.07 ± 1.88	3.50 – 10.00	$n_1 = 15$, $n_2 = 18$ p = 0.677	

GENERAL DISCUSSION

Sexual selection has the power to shape genetic diversity by influencing individual mating success dependent on quality (Neff & Pitcher 2005; Lehmann et al. 2007). Most theoretical and empirical studies have concentrated on female preferences for male traits in species with conventional sex roles (e.g. Candolin 2003; Lehmann et al. 2007). In such species, females are thought to be choosy while males compete for access to mates (Eens & Pinxten 2000). Only a small number of studies have simultaneously considered male preferences for female traits (e.g. Amundsen & Forsgren 2001; Berglund & Rosenqvist 2001).

A good example for the role of sexual selection in shaping genetic diversity are the genes of the major histocompatibility complex (MHC/MH), which are involved in the adaptive immune defense (Janeway et al. 2002). Preferences for both MHC diverse mates and specific MHC alleles have been found, and these preferences have been shown to increase individual pathogen resistance (reviewed in Piertney & Oliver 2006). In teleosts, MH-based mate choice has been found in females, but it is unknown whether males are also able to recognize and use these cues during reproduction (e.g. Reusch et al. 2001; Forsberg et al. 2007; Agbali et al. 2010). This thesis investigated the role of sexual selection in shaping MHIIb genetic variation in the sex-role reversed potbellied seahorse (*Hippocampus abdominalis*), a species with male choice and female-female competition (Wilson & Martin-Smith 2007). Sex-role reversed species such as the seahorse, offer a unique opportunity to test the importance of male mate choice on traits of evolutionary significance.

In chapters I and II, I investigated MHIIb gene diversity in the potbellied seahorse. Targeted gene sequencing, genome walking and a 454 transcriptome screen all identified a single expressed copy of the MHIIb gene in this species. The pattern of genetic variation at the seahorse MHIIb locus is typical for vertebrates, with high nucleotide diversity in the peptide binding region (PBR) and much lower levels of variability outside this region (Hughes 2000). The high PBR diversity in the seahorse is maintained by a combination of positive selection and intralocus recombination. Our data suggests that neutral variation outside the PBR is heavily reduced by intralocus gene conversion and a biased nucleotide composition. While several studies have

criticized the role of gene conversion in shaping MHC polymorphism (Klein & Figueroa 1986; Martinsohn et al. 1999; Nei & Rooney 2005), this thesis provides strong evidence that gene conversion and recombination are important mechanisms influencing patterns of MHC genetic diversity.

Despite a general congruence between the pattern of nucleotide diversity found in the seahorse MHIIb gene and that observed in other teleosts, the seahorse shows levels of diversity, especially outside the PBR, that are lower than those observed in other species. Studies on teleosts show that MH gene diversity is highly variable in this group. A recent whole genome sequence (Star et al. 2011) indicates that the genes of the MH class II pathway are completely absent in Atlantic cod (*Gadus morhua*), calling into question the typical paradigm of class I and II MHC loci thought to be ubiquitous in vertebrates. The loss of MH class II loci in *G. morhua* has been accompanied by an expansion of MH class I genes, suggesting that other aspects of the adaptive immune system have compensated for the loss of class II loci. The lack of linkage of MH loci in teleost fishes is thought to increase the evolutionary flexibility of the adaptive immune system (Stet et al. 2003), and may be at least partially responsible for the high levels of intraspecific variation found in this group.

Interestingly, PCR tests of seahorse MHIIb primers in the sex-role reversed broad-nosed pipefish (*Syngnathus typhle*) (A. Bahr, unpublished data), a close relative of seahorses, and a 454 transcriptome screen on *S. typhle* (T. Reusch, in prep.) have as yet found no evidence of MH class II genes in this species. Next-generation sequencing of the seahorse transcriptome (Gauthier et al., in prep.) has identified major components of the innate and adaptive immune system in this group, and has found evidence of multiple MH class I loci in this species. The application of a genome perspective to immune genes of the seahorse, the pipefish and other closely related species, as well as comparisons of the innate immune system between these species could help to shed light on the immune system evolution in this group and to investigate the wider generality of the pattern observed in gadoid fishes.

Previous studies in teleosts have found evidence of MH-based mate choice in females, but not in males (Forsberg et al. 2007; Neff et al. 2008), and the observation of a similar pattern in sex-role reversed species would be expected to relax the importance of sexual selection on MHC loci in such species. The observation that the PBR diversity of the seahorse MHIIb gene is comparable to that detected in species with conventional sex roles was consistent with the idea that natural selection may be sufficient to maintain high levels of PBR diversity. In contrast to our expectations, we found that female seahorses preferred MHIIb dissimilar males, while males preferred large-bodied females. These preferences also influenced realized mating behavior, a pattern consistent with mutual mate choice for different mating cues in this species. These results are striking, and while males were previously thought to be the choosy sex in the seahorse based on behavioral observations, this thesis clearly shows that *H. abdominalis* can no longer be considered a sex-role reversed species. Sexual selection acts on both sexes in this species, with males preferring large-bodied females and females choosing on the basis of MHIIb-compatibility. This pattern of choice has been overlooked in previous studies on syngnathid fishes, which have focused on individual mating cues (e.g. Mattle & Wilson 2009; Ratterman et al. 2009) and/or male preferences (e.g. Sundin et al. 2010; Lindqvist et al. 2011). These two problems are typical of many behavioral studies, and have tended to reinforce the traditional dichotomous view of sex roles. Recent studies incorporating both male and female preferences and multiple mating cues indicate that mutual mate choice likely occurs far more often than previously assumed (see Hooper & Miller 2008 for examples), and should be expected considering the fact that both females and males can achieve important direct and indirect fitness benefits through active choice. In addition to the exploration of the importance of individual mating cues, in order to investigate their absolute effect on choice, future behavioral studies should try to incorporate multiple cues important for both female and male mate choice, to yield a better understanding of how sexual selection acts in natural populations.

As female preferences for MH-dissimilar males likely shapes MHIIb diversity in the potbellied seahorse, this species is not an appropriate model in which to

disentangle the effects of natural and sexual selection on MHC gene diversity. Future studies should investigate other syngnathid species thought to be sex role reversed. If exclusive male choice can be confirmed in these species, current data suggests that MH-based mate choice may be negligible, offering systems in which to separate the effects of natural and sexual selection. Asexual species might also be suitable to address this question. While the asexual Amazon Molly (*Poecilia formosa*) exhibits lower MHIIb diversity relative to a close sexual relative, this difference could not clearly be attributed to sexual selection due to potential differences in demography and/or parasites (Schaschl et al. 2008).

Evidence for MHC-based mate choice by males has only been found in a small number of vertebrate species, including humans and mice (Wedekind & Furi 1997; Penn & Potts 1999). Chapter III of this thesis contributes to an increasing number of studies showing that the use of MH-based olfactory cues during reproduction in teleosts is restricted to females (Forsberg et al. 2007; Neff et al. 2008). Future studies should seek to clarify whether sex-specific physiological differences in the olfactory organs influence male ability to recognize and process these cues. Profound differences in olfactory morphology and ability have been found in mammals (Baum & Keverne 2002; Good & Kopala 2006), and the presence of sex-specific differences in fishes could provide a simple explanation for the lack of male MH-based mate choice in this group. Alternatively, if male teleosts are able to detect these olfactory cues, the lack of MH-based preferences in male mate choice could reflect the lower value of these traits relative to other visual, behavioral or olfactory cues. A third possibility for the observed pattern in teleosts is that an MH-independent signal, which might only be produced by males, may be necessary to validate the MH signal and to allow its detection during mate choice, as suggested in threespined sticklebacks, *Gasterosteus aculeatus* (Milinski et al. 2010). Interestingly, Gillingham et al. (2009) have recently shown that despite the absence of male MHC-based precopulatory mate choice, male red junglefowls (*Gallus gallus*) showed a cryptic preference for MHC dissimilar females by allocating more sperm to these females. Such cryptic choice might be an important mechanism by which

male teleosts could influence trait values in their offspring even in the absence of apparent preferences.

Previous observations of MH-based mate choice in female, but not male teleosts, led to the hypotheses that sex-role reversed species such as the potbellied seahorse would lack MH-based mate choice and show a pattern of MH variation distinct from species with conventional sex roles. The observation of female choice for MH-dissimilarity in *H. abdominalis* was unexpected, but this result is in many ways even more interesting than the original expectation. In demonstrating the existence of mutual mate choice in the potbellied seahorse, this thesis clearly shows how both male and female mate choice can influence sexual selection. Mutual mate choice is likely to be widespread, highlighting the need to extend models of sexual selection beyond their traditional framework.

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